PB-221 481

ENVIRONMENTAL EFFECTS ON TOXAPHENE TOXICITY TO SELECTED FISHES AND CRUSTACEANS

Walter R. Courtenay, Jr., et al

Aquatic Sciences, Incorporated

Prepared for:

Environmental Protection Agency

April 1973

DISTRIBUTED BY:

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U. S. DEPARTMENT OF COMMERCE
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EPA-R3-73-035
April 1973

W73. 11323

Ecological Research Series

PB 221 481

Environmental Effects on Toxaphene Toxicity to Selected Fishes and Crustaceans

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U.S. Environmental Protection Agency

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1 Accession Number: W 73-11323

2 Subject Field & Group: 05C

SELECTED WATER RESOURCES ABSTRACTS
INPUT TRANSACTION FORM

5 Organization
Aquatic Sciences, Inc.,
~~2624 N.W. 2nd Avenue~~
Boca Raton, Florida. ~~33432~~

6 Title
Environmental Effects on Toxaphene Toxicity to Selected Fishes and Crustaceans, (all caps)

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16 Project Designation
~~EPA, WPO~~ Contract 14-12-532. (EPA Project 18080DLR)

21 Note
73-035
Copy available from GPO Sup Doc as EP 1.23/, $1.00 ; microfiche from NTIS as PB 221 481, $0.95.

22 Citation
Environmental Protection Agency, report ~~number~~, EPA-R3-73-035, April 1973. 73p, (Ecological Research Series)

23 Descriptors (Starred First)
*Pesticide Toxicity, *Chlorinated Hydrocarbons, *Salinity, *Temperature, *Dissolved Oxygen, Bass, Mullets, Crabs, Pink Shrimp, Pathology of Pollutants, Larvae, Juvenile *Fish, ~~Florida~~, *Lethal limit, *Crustaceans, Toxicity

25 Identifiers (Starred First)
*Toxaphene, ~~*Interaction~~, *Synergism, Micropterus salmoides, Mugil cephalus, Mugil curema, Trachinotus carolinus, Callinectes sapidus, Penaeus duorarum, Sesarma cinereum, Rhithropanopeus harrisii, Zoea, Megalopa, Mysis, Protozoea, Nauplius

27 Abstract

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Earliest developmental stages of the fish species treated are more resistant to high levels of salinity, and to low levels of dissolved oxygen, but more sensitive to high temperatures than are later stages. Decapod larvae showed increasing tolerance to Toxaphene with increasing developmental age. Synergistic effects between Toxaphene and the three environmental factors were suggested in the species tested. Some histopathology was noted in fry of bass and mullet, and in larvae of S. cinereum, C. sapidus, and R. harrisii. (EPA)

Abstractor: Walter R. Courtenay, Jr. Institution: Aquatic Sciences, Inc.

WR 102 (REV. JULY 1969) WRSIC
SEND, WITH COPY OF DOCUMENT, TO: WATER RESOURCES SCIENTIFIC INFORMATION CENTER U.S. DEPARTMENT OF THE INTERIOR WASHINGTON, D. C. 20240
GPO: 1970 - 407-891

EPA-R3-73-035
April 1973

ENVIRONMENTAL EFFECTS ON TOXAPHENE TOXICITY TO SELECTED FISHES AND CRUSTACEANS

By

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Contract No. 14-12-532
Project 18080 DLR

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Prepared for

OFFICE OF RESEARCH AND MONITORING
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

For sale by the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402
Price $1 domestic postpaid or 75 cents GPO Bookstore

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This report was submitted in fulfillment of Project Number 18080DLR, Contract 14-12-532, under the sponsorship of the Office of Research and Monitoring (formerly Water Quality Office), Environmental Protection Agency.

In requesting information about this report from Aquatic Sciences, Inc. refer to the identification number ASI 216-02.

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CONCLUSIONS

1. Synergistic effects were suggested between temperature-reduced dissolved oxygen and salinity-reduced dissolved oxygen for eggs, larvae and juveniles of fish and in some cases for larvae of decapod crustaceans but were not statistically demonstrable.

2. Synergistic effects have been statistically demonstrated between Toxaphene and all three environmental parameters for some stages of decapod species studied. In other cases, the data suggests synergistic effects.

3. The earliest stages (embryos) in the life history of fishes were most tolerant to extremes of salinity. Sac-fry larvae were more tolerant to this factor than juveniles.

4. Decapod larvae became more tolerant to Toxaphene at later developmental stages than at earlier stages. In some cases a 10-fold increase in tolerance was noted from one stage to the next.

5. Decapod larvae became increasingly tolerant of salinity extremes in advanced stages but the increase was not as striking as the increased tolerance of Toxaphene.

6. Sac-fry of the striped mullet showed enlargement of cells and increased numbers of cells in glandular tissue in the head region when exposed to high concentrations of Toxaphene.

7. Sac-fry of largemouth bass exposed to extreme salinities exhibited deformation of the yolk sac and the notochord. The latter caused a humpbacked condition.

8. Larvae of S. cinereum, C. sapidus, and R. harrisii exposed to extreme concentrations of Toxaphene (<96 hr TL_{50}) showed destruction of the hepatopancreas and constriction of the intestine.

9. Plastic containers should not be used in pesticide bioassays because of the rate and degree of sorption by the plastic. This phenomenon led to gross overestimates of 96 hr TL_{50} values for all fish studies in these tests.

10. Glass containers also exhibit some degree of sorption to pesticide so that the 96 hr TL_{50} is overestimated (10 to 25%). Chemical assays for the pesticide should be made during all bioassay tests.

RECOMMENDATIONS

1. Continued research is recommended on these species to elucidate the effects of Toxaphene and environmental parameters on stages not covered in this report.

2. It is believed that the 96 hr TL_{50} is higher than the TL_{50} ultimate. In the case of fishes, sensitivity seems to be related to development of gills, not included in the test period. In the case of crustaceans, the most sensitive phase in development is the molt between stages. The contract did not call for study at these points in the life history. Further study is recommended to elucidate these points.

3. Since significant synergistic effects of Toxaphene with environmental parameters were noted, consideration must be given to the environmental parameters of receiving waters in determining maximum permissible concentrations of Toxaphene.

4. Exposure to extreme conditions has a marked effect on behavior and feeding of both fish and decapods. Further study is recommended to elucidate the role of starvation as a possible cause of death.

5. The synergistic effect between Toxaphene and salinity suggests that Toxaphene interferes with osmoregulation and perhaps ionic regulation. Studies are recommended to examine this hypothesis.

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INTRODUCTION

For several decades there has been increasing concern among scientists and fishermen (sport and commercial) that aquatic organisms and their population dynamics are being affected adversely through the addition of various chemicals to the aquatic ecosystem. Pesticides, particularly the persistent chlorinated hydrocarbons, have been implicated in many "fish kills" in the United States and elsewhere. These chemical pollutants, along with many others, can be passed through the food chain and may be accumulated to lethal levels in those organisms at the top of the chain through the process of biological magnification (Egler, 1964).

The chlorinated hydrocarbon insecticides generally enter aquatic ecosystems by runoff during periods of rain or may be directly introduced through carelessness during applications. Highest concentrations occur in and near agricultural areas where these pesticides are most often used. Once these chemicals are in aquatic situations, they are distributed downstream into lakes, estuaries, and eventually into marine waters (Nicholson, Grzenda, Laver, Cox, and Teasley, 1964).

Dilution occurs from the site of entry of pesticides into the aquatic ecosystem and continues throughout the downstream drift of these chemicals. High concentrations at the source of entry may produce a fish kill. Similar effects may not occur downstream as the chemicals undergo dilution to sublethal levels. Pesticides typically enter the lower portions of inland waterways, estuaries, and marine waters in sublethal concentrations.

The coastal regions of streams, rivers, canals, and inshore marine waters are generally shallow, often associated with marshes and swamps, and undergo wide fluctuations in hydrographic conditions with tidal, climatic, and seasonal changes. Variations in such characteristics as salinity and temperature are often dramatic in these areas. Variations in temperature are now being produced by man as new electrical power plants are constructed. Many of these are thermonuclear and require vast quantities of water to cool the reactors, and return the heated effluent to the environment.

Coastal regions are very productive in terms of aquatic organisms. Shallow areas of fresh waters, estuaries, and inshore marine habitats provide vast breeding and spawning grounds for the species present. Moreover, these same factors are of economic and recreational importance to man because large commercial and sport fisheries depend upon these areas of stock renewal for the fisheries.

Fishery biologists have long recognized the importance of water quality in these areas to renewal of fishery stocks although until recently relatively few biologists have investigated water quality with respect to chemical pollutants (Holden, 1966; Johnson, 1968; Butler, 1966). While they cite declining stocks of animals to support a fishery, and pollution

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caused fish kills, no one has investigated the less apparent but perhaps more significant effects of sublethal levels of pesticides on the important early life history stages of these organisms. Similarly, there have been few studies on interaction effects of these pesticides and fluctuations in environmental factors for these same life history stages. These problems form the basis of the study reported herein.

Behind the urbanized coastal regions of Florida lies a large agricultural complex. The climate of Florida, particularly that of central and southern regions, provides a twelve month growing period for many fruit and vegetable crops. Citrus fruit is the largest agricultural crop. Around and south of Lake Okeechobee are thousands of square miles which are utilized for vegetable and fruit production. Florida ranks as the third largest cattle-producing state.

It has been estimated that 25 thousand tons of pesticides are used annually in Florida, mostly by agriculture, with only a small amount used for domestic pest control. Prior to 1969, DDT was the most commonly used insecticide for agricultural crops in central and south Florida. During 1969 the use of DDT was restricted and farmers began using Toxaphene instead (James E. Brogden, Department of Entomology, University of Florida, personal communication). Exact figures on the amounts of specific pesticide useage in Florida are not available from any source.

Toxaphene is administered to crops in the same formulations as was DDT. One of the most popular is a mixture of this chlorinated hydrocarbon with an organophosphate insecticide, typically Parathion. It was used in this manner on potatoes until the registry for use of Toxaphene on this crop was cancelled (James E. Brogden, personal communication). This formulation remains in use on corn, beans, cabbage, and for control of caterpillars on domestic lawns. Toxaphene is also used by itself or with Parathion on peanuts, soybeans, southern peas, cabbage, peppers, eggplant, tomatoes (south Florida's major vegetable crop), and early season celery. Toxaphene is the active ingredient of many livestock sprays for beef cattle and is also used for treatment of pastures (James E. Brogden, personal communication).

Toxaphene, therefore, has been one of the most likely chemical pollutants to be found in south Florida waters since 1969, although this was not tested in this study. The U. S. Geological Survey has found DDT in south Florida waters and soils (Benjamin McPherson, personal communication) and this indicates that its replacement, Toxaphene, is probably now present in these same areas. Few data are available on the effects of Toxaphene on aquatic animals. It is for these reasons that Aquatic Sciences, Inc. chose Toxaphene as the insecticide to be tested.

The aquatic animals utilized in this study were chosen because (1) they are distributed in the waters described above, (2) they are important as commercial, sport, or forage organisms, (3) knowledge of their physiology and/or life history is available in the scientific literature, and

(4) these species could be cultured at Aquatic Sciences, Inc. Information on these organisms is provided in the introduction for the fish and crustacean sections of this report.

FISHES

INTRODUCTION

Four species of fishes were chosen for this study. The largemouth bass, Micropterus salmoides (Lacepede), was included because (1) it is the most important freshwater sport fish in the southeastern United States, and particularly Florida, where millions of dollars are expended annually by sportsmen (J. Walter Dineen, personal communication), (2) the species inhabits waters which adjoin or flow through agricultural areas, and (3) largemouth bass spawn and live in fresh waters in the coastal plain and in the upper reaches of estuaries where fluctuations of temperature and salinity and, on occasion, dissolved oxygen (when eutrophication is advanced) occur. With or without the presence of pesticides, these fluctuations can cause stress.

Two species of mullet, Mugil cephalus Linnaeus, the striped mullet, and Mugil curema Valenciennes, the white mullet, were chosen as being representative of estuarine fishes. They provide an important commercial fishery in Florida with an annual value exceeding $2.4 million (Anon., 1970). Both species spawn at sea, the late larvae and early juveniles returning to inshore waters at a standard length of between 14 and 20 mm. This life history stage coincides with a major change in the feeding mechanism and a departure from a zooplankton - harpacticoid copepod diet to filter feeding on detrital material, primarily of plant origin, in bottom sediments (Cowart, 1971). At this point, however, the two species diverge in their habitat preferences. Mugil cephalus juveniles enter intracoastal waterways, canals, and rivers, into fresh waters such as Lake Okeechobee inhabited by largemouth bass and other freshwater fishes. Juveniles of M. curema migrate into low salinity waters of intracoastal waterways, canals, and estuaries but typically do not invade waters of less than 15 ^{o}/oo salinity.

Trachinotus carolinus (Linnaeus), the Florida pompano, was chosen as a representative marine species for this study. From an economic standpoint, T. carolinus is the most valuable commercial food fish along the Atlantic coast of the United States and represents a $1 million annual fishery in Florida alone. Mariculture interests have placed Florida pompano as the high priority species for commercial culture.

Although the entire life history of T. carolinus has not been thoroughly studied, adults apparently spawn at sea. Early juveniles return to inshore waters at a standard length of 10 to 20 mm where they are often found in the surf zone along beaches (Cowart, 1971).

Three early life history stages of each species were studied, gastrula, 10-12 day post hatch larva, and juveniles of 20-30 mm standard length. Gastrulae hatched during the 96 hour exposure period, and reached a late sac-fry stage by the end of the test period. These stages were chosen

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because of the relative differences in developmental complexity and organogenesis. These stages are less able to move from one area to another during periods of environmental stresses than are their parent stocks.

The early life history stages of all four species share several further important considerations for inclusion in this study: (1) they are particularly critical stages in development, (2) at least one or more of these stages occur in areas where interaction effects between environmental factor fluctuation and man-induced environmental pollutants, such as Toxaphene, can occur (3) methods for laboratory culture of these fishes for use in research have been and continue to be developed and improved at Aquatic Sciences, Inc., and (4) all four species are of great economic value in Florida.

MATERIALS AND METHODS

Micropterus salmoides eggs are demersal and were collected attached to rocks from Conservation Area II, Broward County, Florida. Eggs at the late gastrula - neurula stage marked by the first appearance of melanophores were selected for testing. Other eggs were allowed to develop and hatch to produce the sac-fry used in testing. Some additional sac-fry were obtained from the Federal Fish Hatchery, Welaka, Florida. Juveniles were obtained from two sources; Federal Fish Hatchery, Welaka, Florida, and Florida Game and Freshwater Fish Commission Hatchery, Richloam, Florida. Additional juveniles used only for screening tests were derived from Federal Fish Hatcheries in Georgia, West Virginia and South Dakota.

Adult *Mugil cephalus* were collected by cast net at Boynton Beach Inlet, Palm Beach County, Florida. These adults were then spawned in the laboratory to produce a limited number of eggs. Juvenile *M. cephalus* were collected by seining in Indian River, St. Lucie County, Florida and Tampa Bay, Hillsborough County, Florida.

Mugil curema juveniles were collected from the same locations as *M. cephalus*, the majority at Indian River, Florida.

Trachinotus carolinus juveniles were collected at Cocoa Beach, Brevard County with limited numbers being derived from Daytona Beach, Volusia County, Florida and St. Augustine, St. Johns County, Florida.

Aquatic Sciences, Inc. has conducted considerable supportive research directed toward manipulation of unripe fishes into prespawning condition, spawning and rearing. This is the only procedure available for obtaining adequate numbers of eggs and larvae of *M. cephalus*, *M. curema*, and *T. carolinus* for experimentation.

The original proposal (ASI Document Number 103-02) and the Schedule for Contract 14-12-532 both specify that tests with eggs will begin at the onset of gastrulation (embryonic shield to primitive streak). Several problems were encountered in attempting to test eggs at this stage. First, there is great variability in the number of unfertilized or dead eggs either collected from the field or produced in Federal hatcheries or our own laboratory. For *M. salmoides*, from less than 1% to 60% of the eggs in a nest were dead or unfertilized. These eggs must be removed prior to testing. Second, embryos are encountered which, regardless of source, terminate development because of processes not fully understood. Arrested development usually occurs during early cleavage to blastula stages and to a lesser extent in later stages. The proportion of eggs in a given batch which exhibit this phenomenon is highly variable. These eggs cannot be identified and removed at the early gastrula stage. These biological anomalies could lead to large errors in experimental results.

It was therefore necessary to select embryos of each species for testing at a slightly later stage of development than specified in the contract. When embryos are selected at the late gastrula - neurula stage after melanophores become visible, nearly 100 percent of the selected embryos hatch when maintained under suitable conditions. This stage can be readily distinguished from unfertilized eggs, arrested embryos, and other developmental stages. Therefore embryos were selected at this stage for all tests.

Acquisition of larvae from field or hatchery operations does not present the same problems as are inherent with the eggs, at least with regard to M. salmoides. Our experience has been excellent in obtaining healthy fishes from both sources. Only two minor problems were encountered and only one of these could probably be reduced through laboratory culture of this species. The first problem is that certain larvae from a given spawning could be referred to as the "runts of the litter"; i.e., they do not grow as rapidly as the majority, and therefore are subject to heavier pressures of competition by their siblings leading to death by starvation or cannibalism. The second problem is a correlary of the first. Whenever possible, hatchery personnel attempt to select larvae of the same size. In doing this larvae of different ages may be selected, and occasionally "runts" from an older group of larvae may be selected along with the average of a younger group because of their similar size. In some cases it appears that these "runts", in spite of their equality in size, are still unable to compete with the younger individuals and may starve to death or become victims of cannibalism.

Juveniles are subject to injury during collection and transport from field to laboratory. In addition they may be diseased when collected. Therefore all juvenile fishes were quarantined for 24 to 48 hours prior to experimentation. Only juveniles from collections exhibiting low mortality during this period were used in experiments.

With one exception, all acute and interaction determinations were conducted utilizing styrofoam fish shipping boxes manufactured by Plasti-Kraft of Ozona, Florida and lined with a 15 x 15 x 22 inch 0.004 mil polyethylene bag. This apparatus was selected after considerable testing based on the following criteria: (1) containers should provide adequate and uniform life support for experimental fishes without filtration for a period of 96 hours and with filtration (i.e., corner filter) for long term exposures; (2) containers should be of convenient size for handling, maintenance, and storage; (3) containers should have better insulation qualities than glass aquaria; (4) containers should be readily sealed for reduced oxygen experiments; (5) containers should be inexpensive and disposable. Few of these criteria are met by the glass containers (glass pickle jars) routinely used in bioassay experiments or glass aquaria. It is widely recognized that many, if not all, of the chlorinated hydrocarbon pesticides will adhere to glass and, in some cases, will chemically bind to the glass. The use of a polyethylene liner in a styrofoam "frame" does not reduce chlorinated hydrocarbon adsorption to the container surface but it does provide for a low cost,

disposable liners which is destroyed after each test with Toxaphene. Each apparatus contained twenty liters of water. Water circulation and oxygen saturation were maintained with a single 1 inch air stone in a corner of the unit.

The experimental apparatus utilized for juvenile T. carolinus was different than that described above. Two containers were tested with T. carolinus: (1) styrofoam fish shipping boxes with plastic liners containing 20 liters of water and (2) large cylindrical polyethylene vessels with plastic liners containing 80 liters of water. Survival rates were examined in these containers with and without filtration for 5, 10, 15, 20, and 25 fish per tank and several different feeding regimes including live adult Artemia and Tetramin® flake food. Juvenile pompano prefer live adult Artemia although they readily accept Tetramin®. They feed selectively on the latter diet, rejecting the green flakes altogether. Water samples were tested for pH and ammonia content at the end of 96 hours (Table 1). Based on these tests, the large plastic lined polyethylene container without filtration, but with an airlift device for aeration and water circulation, was selected.

TABLE 1.

WATER QUALITY DATA FOR TWO EXPERIMENTS DESIGNED TO SELECT CULTURE CONDITIONS FOR TESTS INVOLVING JUVENILE Trachinotus carolinus

A - 20 liters volume

				July 2		July 3		July 6	
Tank	Food	Filtration	Animals/Tank	pH	NH_3	pH	NH_3	pH	NH_3
1	Tetramin®	with	25	7.1	9.37	7.3	14.3	7.8	30.3
2	Artemia	with	25	7.3	7.40	7.5	10.6	7.8	20.5
3	Tetramin®	without	25	7.3	9.37	7.4	12.1	---	----
4	Artemia	without	25	7.5	6.25	7.6	10.5	7.9	19.8
5	Tetramin®	without	0	7.1	0.75	7.8	1.94	7.9	8.1

B - 20 liter versus 80 liter

				13 April		19 April	
Tank	Volume	Animals/Tank	Filtration	pH	NH_3	pH	NH_3
1	80	25	without	7.6	1.62	7.4	2.42
2	80	20	without	8.0	1.21	7.4	1.54
3	80	15	without	7.9	0.90	7.5	0.82
4	80	10	without	7.8	0.59	7.4	0.57
5	80	5	without	7.9	0.45	7.3	0.53
6	80	25	with	7.9	0.57	7.5	0.38
7	80	20	with	7.9	0.49	7.4	0.45
8	20	15	without	7.6	6.25	7.2	9.37
9	20	10	without	7.7	2.21	7.6	6.25
10	20	15	with	7.7	6.70	7.3	7.85
11	20	10	with	7.7	5.16	7.6	8.10

Fertilized eggs and sac-fry were placed in specially designed "protectors" during exposure for ease of handling, examination, feeding, and removal of dead individuals. The "protectors" consist of cylindrical baskets of fine mesh netting supported by cylindrical PVC frames and floated from a styrofoam collar. The collar is inexpensive and disposable.

In standard bioassay tests, experimental animals are not fed during exposure. Food deprivation is suggested to reduce the number of deaths resulting from breakdown products of non-ingested food materials and from increases in metabolites from experimental animals (Anon. 1960). While these factors may be real problems in bioassays, food deprivation is an unnatural situation and in itself becomes a stress factor. Furthermore, larval and juvenile fishes of the sizes used in this project cannot be maintained for 96 hours without feeding.

Therefore, all experimental animals were fed according to the following procedure: three times daily food was added until the fish ceased feeding, usually 15 to 20 minutes. Excess food was then removed. Micropterus salmoides larvae were fed Artemia nauplii and juveniles were fed live Daphnia, M. cephalus and M. curema were fed live Cyclops, and T. carolinus were fed live adult Artemia (when live Artemia was not available, either frozen Artemia or, rarely, Tetramin® was substituted). Chemical monitoring of the water at the end of each experiment rarely showed significant metabolite concentrations.

Twenty-five fish in each "protector" is the optimal number for adequate survival. There are many problems associated with counting very small, live, active fishes and consequently several individuals fewer or more than the desired number were frequently introduced into "protectors". In addition some cannibalism was observed. Cannibalism occurred at a variable rate which could not be precisely determined in every case.

Because of potential counting errors in placing fish in the "protectors" and variable amounts of cannibalism during tests, the initial n (number of experimental animals) is taken to be the number of dead recorded and preserved at each observation time plus the number of animals alive at the termination of an experiment. The number dead was recorded at the end of each 24 hour period in the test. Thus, if only 23 fish can be accounted for in a given "protector" at the end of the experiment (total dead and live), percent mortality is based on this number. This procedure excludes the effect of cannibalism on the mortality data. The fact that the cannibalistic individual(s) may survive an exposure better than others because of this ready source of food is, however, masked.

The procedures followed here effectively cancel out mortality resulting from predation both by other animals and by cannibalism which together probably represent the major cause of death in natural populations. This still leaves the "natural mortality syndrome" of inexplicable deaths of animals maintained under "optimal" conditions. The procedure for apportioning animals in the several conditions of each test did not completely

exclude bias from this source because it did not insure complete randomization.

Temperature Experiments

In temperature tests, temperature was controlled by thermoregulated heaters. Over the range of temperature to be tested, experimental units were maintained at 5°C increments. Salinity was maintained constant and oxygen at saturation. The range tested for each species is given in Table 2.

Salinity Experiments

In salinity experiments, tests were conducted at 5 °/oo increments over the selected range for each species. Water of the desired salinity was prepared by dilution of artificial seawater (Instant Ocean Synthetic Sea Salts®, Aquarium Systems, Inc.) with tap water. If salinities higher than the stock solution were required, Instant Ocean Synthetic Sea Salts® were added. Salinity was measured with a hydrometer to ±0.5 °/oo. Temperature was kept constant at 23-24°C, oxygen at saturation. Test ranges are given in Table 2.

Reduced Dissolved Oxygen Experiments

Four levels of dissolved oxygen were tested, 10, 30, 75, and 100% saturation. The oxygen dilution systems to produce reduced dissolved oxygen levels consisted of 1 quart mixing bottles with separate air and nitrogen inflows monitored by manifold valves and flowmeters. Air from the mixing bottles was then diffused directly into the water in experimental tanks through 1 inch airstones. Water in experimental tanks was maintained free from contact with oxygen-saturated air by closing the plastic liners and providing an air vent. Oxygen concentration was monitored with a Galvanic Cell Oxygen Analyzer (Precision Scientific) and Winkler titrations.

Toxaphene Experiments

Toxaphene used in both the fish and crustacean portions of this project was obtained in two forms from Southern Mill Creek Products Company, Inc., of Tampa, Florida. The first form, used only in preliminary screening experiments, has the trade name of Toxaphene EM-6 and contains 60% Toxaphene (chlorine content 67-69%), 35% xylene-range aromatic hydrocarbon solvent, and 5% inert ingredients. This is called 6 pound per gallon Toxaphene.

The second form, which is presently utilized in all acute and interaction tests, Toxaphene EM-8, contains 71.6% Toxaphene (chlorine content 67-69%), 23% xylene, and 5.4% inert ingredients, and is called 8 pound per gallon Toxaphene. Only one lot was used throughout this research. No analyses for Toxaphene were required under the contract. The methods for preparation of stock and experimental solutions developed by Mahdi (1966) were followed. Test concentrations for each test are summarized in Table 2.

TABLE 2.

RANGES OF TEMPERATURE, SALINITY, REDUCED DISSOLVED OXYGEN AND CONCENTRATION LEVELS OF TOXAPHENE TESTED FOR EACH SPECIES AND STAGE

Temperature		(°C)
Micropterus salmoides	embryo	25 - 39
	sac-fry, juvenile	25 - 40
Mugil cephalus	embryo	22 - 38
	juvenile	25 - 40
Trachinotus carolinus	juvenile	26 - 39
Salinity		(°/oo)
Micropterus salmoides	all stages	0 - 20
Mugil cephalus	embryo	0 - 65
	juvenile	0 - 68
Trachinotus carolinus	juvenile	0 - 60
Reduced Dissolved Oxygen		(%)
Micropterus salmoides	embryo	10 - 100
	sac-fry	30 - 100
Mugil cephalus	embryo	30 - 100
Trachinotus carolinus	juvenile	30 - 100
Toxaphene		(ppm)
Micropterus salmoides	embryo	0.005 - 5.00
	sac-fry	0.010 - 0.10
	juvenile	0.010 - 0.25
Mugil cephalus	embryo	0.005 - 0.50
	juvenile	0.005 - 0.50
Trachinotus carolinus	juvenile	0.010 - 0.25

Upon completion of the Toxaphene bioassay tests, a contract modification was negotiated to determine the concentration of Toxaphene in simulated bioassay tests without animals. Three concentrations were prepared in the same way as for bioassay tests in the polyethylene-lined styrofoam boxes. Samples were removed for analysis at 3 hr., 48 hr., and 96 hr. after solution preparation. The samples were then extracted with hexane, and analyzed by gas chromatographic methods.

Since the recovery of Toxaphene was very low, a subsequent experiment was conducted to develop a Toxaphene budget for the system at a single concentration. The solution was prepared at 0.5 ppm in the usual manner. A sample of the solution was taken at 3 hr. and 24 hr., extracted with hexane, and analyzed. The remaining solution was then drawn off carefully so that any precipitate would not be removed. The container was then rinsed with distilled water to remove particulate Toxaphene. The wash was extracted with hexane and analyzed. The container was then washed briefly with hexane to remove adsorbed Toxaphene and analyzed. That portion of the bag exposed to the solution was then cut into small pieces and extracted with hexane for twenty-four hours to remove absorbed Toxaphene. The hexane partially dissolved the plastic but this did not interfere with the gas chromatograph analysis. It was not possible to re-extract the plastic; therefore the completeness of recovery of absorbed Toxaphene is not known.

Interaction Tests

Five combinations of factors were tested: temperature-reduced dissolved oxygen, salinity-reduced dissolved oxygen, temperature-Toxaphene, salinity-Toxaphene, and reduced dissolved oxygen-Toxaphene. Conditions were established and maintained as described above for each parameter.

Levels to be tested were selected to be sublethal based on the results of the acute tolerance tests, ranging from an optimal level to just below the 96 hr TL_{50} level.

In acute and interaction tests involving eggs and sac-fry, there were 4 replicates at each test level or a total of about 100 individuals at each test level. For juveniles there were 2 replicates at each test level or approximately 50 individuals.

96 hr TL_{50} values were determined by the graphic method of Douderoff et al. (1951, Anon, 1965) with the data for replicates pooled.

Specimens from each test series were preserved for examination of gross morphology and histopathology. Two hundred ninety-two specimens from tests involving Toxaphene were sectioned at 6 μ and stained with hematoxylin-eosin for histological study of gill, pharyngeal region, digestive tract, liver, kidney, and epithelial tissues to determine if Toxaphene affects these tissues.

All Toxaphene tests were conducted in completely independent laboratory modules located outside of the main building complex at Aquatic Sciences, Inc. These research spaces were provided to prevent contamination of culture, life support, and other research areas in the main complex.

RESEARCH RESULTS

All three life history stages of Micropterus salmoides were included in acute and interaction exposures. The 96 hr temperature TL_{50} for all stages of M. salmoides ranged from 31.5°C for embryos to 37.2°C for juveniles. The 96 hr salinity TL_{50} for embryos was not established at levels up to 20 °/oo but was 15.8 °/oo for larvae and 12.5 °/oo for juveniles. The majority of embryos tested at 20 °/oo salinity were deformed at hatching. The 96 hr reduced dissolved oxygen TL_{50} ranged from 15.3% for embryos to 47.5% saturation for juveniles. These data are provided in Table 3.

TABLE 3.

96 hr TL_{50} VALUES FOR EACH STAGE OF EACH FISH SPECIES

Species	Stage	Temperature (°C)	Lower Salinity (°/oo)	Higher Salinity (°/oo)	Reduced Dissolved Oxygen (% sat.)
Micropterus salmoides	embryos	31.5	Does not exist	20.0	15.3
	sac-fry	33.5	0.0	15.8	48.0
	juveniles	37.2	0.0	12.5	47.5
Mugil cephalus	embryos	26.4	-	-	-
	juveniles	36.5	0.0	62.0	49.0
Trachinotus carolinus	juveniles	36.2	0.0	50.3	18.0

The temperature-reduced dissolved oxygen interaction test was conducted on larval M. salmoides. Highest mortality occurred at a temperature of 33°C (Table 4). An interaction test between salinity and reduced dissolved oxygen was conducted for both larvae and juveniles. Larvae experienced total mortality at a salinity level of 12 °/oo when oxygen concentration was 75% saturation (Table 4).

Because of problems encountered in acquiring larval Mugil cephalus and in obtaining sufficient numbers of embryos, only one test was conducted on embryos. All tests required for juvenile M. cephalus were completed.

TABLE 4.

PERCENT MORTALITY AFTER 96 HOURS IN INTERACTION TESTS FOR Micropterus salmoides

Sac-fry Larvae

		Temperature (°C)			Salinity (°/oo)		
		22-24	30	33	0	9	12
Reduced Dissolved Oxygen (% sat.)	75	*	13.6	88.0	___	40.0	100.0
	100	8.3	___	___	17.8	___	___

Juveniles

		Salinity (°/oo)		
		0	9	12
Reduced Dissolved Oxygen (% sat.)	75	___	40.0	58.0
	100	87.0	___	___

* - not tested

The 96 hr temperature TL_{50} for M. cephalus was 26.4°C for embryos and 36.5°C for juveniles. A 96 hr lower salinity TL_{50} was not observed for juveniles but a 96 hr upper salinity TL_{50} occurred at 62 °/oo. The 96 hr reduced dissolved oxygen TL_{50} for juveniles was established at 49% saturation. These results are summarized in Table 3.

No interaction tests or long term exposures were conducted with M. cephalus.

Juveniles of Mugil curema were utilized in interaction and long-term exposures. Highest mortalities were experienced in test series with 36°C in temperature-reduced dissolved oxygen exposures, with 60% salinity in salinity-dissolved oxygen tests (Table 5).

TABLE 5.

PERCENT MORTALITY AFTER 96 HOURS IN INTERACTION TESTS FOR Mugil curema*

Juveniles

		Temperature (°C)				Salinity (°/oo)			
		23-24	24	33	36	35	54	57	60
Reduced Dissolved Oxygen (% sat.)	75	___	___	41.2	44.0	___	21.0	24.3	56.0
	100	31.3	24.2	___	___	31.5	___	___	___

A 96 hr temperature TL_{50} of 36.2°C was established for juvenile T. carolinus. A 96 hr lower salinity TL_{50} was not observed for juveniles but a 96 hr upper salinity TL_{50} of 50.3 °/oo was determined. The 96 hr reduced dissolved oxygen TL_{50} for juveniles was 18% saturation. These data are summarized in Table 3.

In interaction exposures, highest mortalities among juvenile T. carolinus were observed at 30% saturation of dissolved oxygen in the temperature-reduced dissolved oxygen and salinity-reduced dissolved oxygen. These data are reported in Table 6.

TABLE 6.

PERCENT MORTALITY AFTER 96 HOURS IN INTERACTION TESTS FOR Trachinotus carolinus

Juveniles

		Temperature (°C)			Salinity (°/oo)			
		22	32	35	33	42	45	48
Reduced Dissolved Oxygen (% sat.)	30	___	72.0	100.0	___	53.0	76.0	75.0
	75	___	4.0	6.0	___	23.0	18.0	28.0
	100	2.0	___	___	2.0	___	___	___

Toxaphene Concentrations in Bioassay Tests

No results of tests involving Toxaphene are reported because the methodology used was subsequently found to be inadequate for these tests. The use of plastic for test containers was shown to result in virtually complete removal of Toxaphene from the system. Therefore TL_{50} values were grossly overestimated and of no value.

The results of the analysis of Toxaphene over time in simulated bioassay tests are presented in Table 7. In both fresh and salt water, the concentration at 3 hr. after solution preparation was in all but one case less than 13% of the calculated concentration. After 48 hours only traces of Toxaphene were measured in all but one case.

TABLE 7.

ASSAYED TOXAPHENE CONCENTRATION IN SIMULATED BIOASSAY TEST WITH PLASTIC CULTURE VESSELS

Water Type	Nominal Concentration (ppm)	Calculated Concentration (ppm)	Assayed 0	Concentration	(ppm) on Day 4
Salt Water	0.50	0.479	0.062	< 0.001	<0.001
30 °/oo	0.10	0.096	0.037	< 0.001	< 0.001
	0.05	0.048	0.0056	< 0.001	< 0.001
Fresh Water	0.5	0.479	0.062	< 0.013	< 0.001
	0.05	0.048	0.001	< 0.001	< 0.001

In a separate experiment we attempted to account for all Toxaphene introduced. This test was terminated at 24 hours and estimates were made of the Toxaphene in solution, in solid form, adsorbed to the plastic and absorbed to the plastic (Table 8). There was a Toxaphene concentration in solution of 0.0274 ppm at 24 hours. The total Toxaphene in solution was 132.7 µg. There was 24.1 µg in the distilled water (particulate fraction). The plastic accounted for most of the Toxaphene with 280.3 µg adsorbed and 319.0 µg absorbed. The total amount of Toxaphene accounted for was 756.1 µg or 39.5% of the amount introduced. The Toxaphene unaccounted for is believed to be associated with the residual undissolved but liquified plastic after decanting the hexane fraction from the extract to determine the absorbed fraction; i.e., the absorbed fraction was underestimated.

TABLE 8.

TOXAPHENE BUDGET AT 24 HR IN SIMULATED BIOASSAY TEST WITH PLASTIC CULTURE VESSELS

Fraction	µg
soluble	132.7
particulate	24.1
adsorbed	280.3
absorbed*	319.0
TOTAL	756.1

µg Toxaphene introduced 1916 µg
% recovered 39.5%

*extraction not complete

Histopathology

The species, stage, test condition, and number of specimens sectioned is summarized in Table 9. Morphological anomalies were noted among embryos of Micropterus salmoides and Mugil cephalus exposed to test situations. The majority of the larvae of M. salmoides which hatched from eggs exposed to 20 °/oo salinity were deformed in four ways: (1) curvature of the notochord causing a "humpback" condition; (2) shortened trunk region in several fry apparently resulting from lack of several embryonic somites; (3) distortion in the shape of the yolk sac in some fry; and (4) forward displacement of the yolk sac in others. As these anomalies did not occur in other newly-hatched fry of the same age from the same test series exposed to lower levels of salinity nor in those of the control series, these anatomical deformations are attributed to the higher salinity.

Histopathological effects were found in the head region of Mugil cephalus embryos exposed in a preliminary Toxaphene 96 hr TL_{50} test. The maximum Toxaphene concentration was 0.5 ppm in this series. Glandular structures in the optic region of those embryos exposed to the highest concentrations of Toxaphene were larger and more numerous than those exposed to lower concentrations and in the control series. These glandular structures appear to be mucous glands and their anomalous condition may be due to epithelial irritation by Toxaphene.

Histopathological effects were also noted in larvae of Micropterus salmoides. Necrosis was found in kidney tissues and the lining of the digestive tract of larvae which had been exposed for a period of 14 days to 10% of the 96 hr TL_{50} for Toxaphene administered in the food. Necrosis of kidney tissues was severe with near total destruction of the kidney tubules. There was no significant destruction of other organs detected.

TABLE 9.

SUMMARY OF FISH SPECIMENS EXAMINED
FOR HISTOPATHOLOGICAL STUDY

Species	Stage	Test Condition	Level	Number of Animals Examined
Micropterus salmoides	larva	Toxaphene	0.000 ppm	6
		Toxaphene	0.001 ppm	2
		Toxaphene	0.1 ppm	2
		Toxaphene/temperature	0.05 ppm/33°C	3
		Toxaphene/salinity	0.01 ppm/9 °/oo	4
			0.05 ppm/9 °/oo	6
			0.05 ppm/12 °/oo	9
		Toxaphene/reduced dissolved oxygen	0.01 ppm/75%	3
			0.05 ppm/75%	3
	juvenile	Toxaphene	0.00 ppm	3
		Toxaphene/temperature	0.01 ppm/35°C	2
			0.05 ppm/35°C	4
		Toxaphene/salinity	0.05 ppm/12 °/oo	4
		Toxaphene/reduced dissolved oxygen	0.5 ppm/75%	5
Mugil curema	juvenile	Toxaphene (14 day)	0.033 ppm	2
Mugil cephalus	egg	Toxaphene	0.00 ppm	13
		Toxaphene	0.5 ppm	15
	juvenile	Salinity	0.00 ppm	2

TABLE 9 (continued)

Species	Stage	Test Condition	Level	Number of Animals Examined
		Toxaphene (14 day)	0.033 ppm	2
Trachinotus carolinus	juvenile	Temperature	24°C	4
		Toxaphene	0.03 ppm	2
		Toxaphene	0.25 ppm	6
		Toxaphene (14 day)	0.0035 ppm	2
		Toxaphene (14 day)	0.017 ppm	2
		Toxaphene (14 day, in food)	0.033 ppm	2
		Toxaphene/temperature	0.01 ppm/35°C	4
		Toxaphene/reduced dissolved oxygen	0.1 ppm/30%	4
		Toxaphene/salinity	0.1 ppm/48 °/oo	2

No morphological or histological effects were observed in specimens exposed to other experimental variables. Further, no histopathological effects were observed in T. carolinus exposed to any experimental variable.

DISCUSSION

Mugil cephalus juveniles were most tolerant of elevated salinity (TL_{50} = 62 °/oo) followed by Trachinotus carolinus juveniles (TL_{50} = 50.3 °/oo. Juveniles of all those species tested tolerated fresh water (TL_{50} = 0.0 °/oo). Micropterus salmoides showed decreasing tolerance of elevated salinity with increasing developmental age (TL_{50} = 20 °/oo for embryos, 15.8 °/oo for larvae and 12.5 °/oo for juveniles).

The results of temperature tests are somewhat different. None of the juveniles used in tests were acclimated to laboratory temperatures of 25°C for more than 48 hours before these fishes entered temperature tests. The results for juveniles are in rather close agreement (96 hr TL_{50} for M. salmoides = 37.2°C; for M. cephalus = 36.5°C; and for T. carolinus = 36.2°C). This coincides well with results for upper temperature limits of many other warm acclimated organisms (Prosser, 1961). Larvae of M. salmoides are not as resistant to higher temperatures (96 hr TL_{50} = 33.5°C) and bass embryos are even less tolerant (96 hr TL_{50} = 31.5°C). Therefore, temperature tolerance increases with development from embryos to juveniles. This is highly significant with respect to injection of thermal effluents into areas in which fishes spawn, i.e., estuaries and bays.

Juveniles of M. salmoides and M. cephalus and larval M. salmoides showed close agreement in dissolved oxygen tests with 96 hr TL_{50}'s of 47.5%, 49%, and 48% saturation respectively. Embryos of Micropterus were most resistant to low levels of oxygen with a 96 hr TL_{50} of 15.3% saturation. Juvenile T. carolinus were quite resistant to reduced oxygen with a 96 hr TL_{50} of 18% saturation. It was noted that their activity during these tests slowed as oxygen content of the water was lowered. The gills of juvenile T. carolinus are probably more efficient in removing oxygen from water than are those of the other species, perhaps representing an adaptation to pelagic life.

The results of the chemical assay tests indicate that Toxaphene is rapidly removed from the test solution by the container. After as little as 3 hours only 13% is in solution and after 48 hours only a trace. This means that all 96 hr TL_{50}'s were grossly overestimated because virtually no Toxaphene was present during the majority of the test. The rapidity of removal precludes calculation of valid TL_{50} estimates for any time interval.

Interaction between sublethal levels of paired test factors was suggested in many if not all combinations tested. This conclusion is based on the fact that mortality for the combined action of variables at levels less than that required to produce 50% mortality (i.e. the 96 hr TL_{50}) was greater than 50% and in several cases was 100%. However the incomplete factorial design used precludes valid statistical treatment.

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If synergistic interactions do occur between environmental parameters as suggested here, then 96 hr TL_{50} values provide only a guideline to the lethal and sublethal levels for the given set of exposure conditions tested and cannot be applied to situations where environmental levels are more or less stressful. Single factors rarely play a dominant role in nature. Therefore bioassay tests should include realistic levels of both natural and man-made parameters in multiple factor interaction designs to measure the credibility of results when applied to the natural environment.

Most tests for each species were performed with specimens from a single geographic location and all specimens in any single test were from a common source. In subsequent studies, it should be determined whether there are differences in tolerance to test parameters for animals derived from several geographic locations.

CRUSTACEA

INTRODUCTION

The purpose of this research was (1) to determine the median tolerance limit of larvae of selected decapod crustaceans for extremes of temperature, salinity and reduced dissolved oxygen concentration and for the pesticide Toxaphene, (2) to determine the interaction effect of pairs of the above parameters at sublethal levels (hereinafter called interaction tests), and (3) to detect histopathological effects at acute and sublethal concentration levels, especially for Toxaphene-exposed animals.

The species selected for study were the blue crab, Callinectes sapidus; the mud crab, Rhithropanopeus harrisii; the drift-line crab, Sesarma cinereum; and the pink shrimp, Penaeus duorarum. Callinectes sapidus and P. duorarum are commercially important species with major fisheries throughout most of their ranges along the Atlantic coast of North America. Rhithropanopeus harrisii and S. cinereum were selected for study for two reasons; they are both ecologically important in their respective habitats, and their larvae are better known physiologically than perhaps any other species.

All larval stages for each of these species have been described in the literature. Callinectes sapidus has 7 (occasionally 8) zoeal stages and a megalopa (Costlow and Bookhout, 1959). The four zoeal and one megalopal instars of R. harrisii were described from plankton specimens by Connolly (1925) and verified in culture by Chamberlain (1962) and Costlow, Bookhout and Monroe (1966). Sesarma cinereum has 4 zoeal and 1 megalopal instar in culture (Costlow and Bookhout, 1960). The naupliar, protozoeal, and mysis stages of Penaeus duorarum were described by Dobkin (1961) from planktonic specimens and verified by Ewald (1965) from laboratory-reared specimens.

The development of culture techniques has made available sufficient numbers of larvae for studies of larval ecology and physiology. Costlow (1967) examined the effects of temperature and salinity on development of the megalopa of C. sapidus but was unable to perform similar studies with the zoeal instars. Results of a complete study of the effects of temperature and salinity on all larval stages are available for S. cinereum (Costlow, Bookhout, and Monroe, 1960) and R. harrisii (Chamberlain, 1962; Costlow, Bookhout, and Monroe, 1966). In addition, studies have been made concerning the effect of eyestalk extirpation on these and other species for the larval stages (Costlow, 1966, 1968). Some data is available on free amino acids in C. sapidus and R. harrisii larvae (Costlow and Sastry, 1966), respiration for Uca, (Vernberg and Vernberg, 1964), and osmoregulation (for Cardisoma, Rhithropanopeus, and Libinia, (Kalber and Costlow, 1966, Costlow and Kalber, 1968) and Sesarma (Kalber and Costlow, unpublished). No data is available in the literature on the internal morphology-histology of larvae of any species except the hermit crab, Pagurus annulipes (Thompson, 1903).

MATERIALS AND METHODS

Ovigerous females of each brachyuran species were collected locally in south Florida and placed in aquaria until the eggs hatched. Sesarma cinereum were collected at Boca Raton, Palm Beach County, Florida, C. sapidus from the St. Lucie Estuary, Martin County, and R. harrisii from drainage ponds on the University of Miami Campus, Dade County, Florida. The females were maintained at 25°C, and a salinity comparable to that of their natural habitat (C. sapidus, 30 °/oo; R. harrisii, 25 °/oo; S. cinereum, 25 °/oo). Hatching occurred within a week after placing in hatching tanks. The adults were not fed during incubation of the eggs.

Larvae were maintained in the laboratory under standardized conditions until the desired stage was reached. Callinectes sapidus larvae were kept at 25 °/oo or, recently, 30 °/oo in 20.3 cm finger bowls and fed sea urchin blastulae-gastrulae and Artemia nauplii. In some recent experiments, Dunaliella salina was provided as food for zoea stage I and II. Sesarma cinereum larvae were maintained at 25°C, 25 °/oo, R. harrisii at 25°C, 25 °/oo. Both species were fed Artemia nauplii. The shrimp, P. duorarum, larvae were purchased at the desired stage (nauplius, protozoea, or mysis) from Seafarms, Inc., Key West, Florida. They were fed with the motile chlorophytan Dunaliella salina in the protozoeal stages and with Artemia nauplii during the mysis stage. The nauplii are non-feeding stages.

All experiments were conducted in 11.4 cm finger bowls. Bowls were examined daily for deaths and molts. The food organisms were provided as needed to maintain adequate food concentrations.

Temperature Experiments

In experiments with temperature as the experimental variable, the cultures were maintained in BOD boxes. Over the range of temperature to be tested, cultures were maintained at 5°C increments. Salinity was kept constant at the culture salinity used for each species. The range tested for each species is given in Table 10.

Salinity Experiments

In salinity experiments, tests were conducted at 5 °/oo intervals over the selected range for each species. Water of the desired salinities was prepared by dilution of artificial seawater (Instant Ocean Synthetic Sea Salts®, Aquarium Systems, Inc.) with distilled water. The stock solution had a salinity of 35 °/oo. To prepare medium with a higher salinity than stock, Instant Ocean Synthetic Sea Salts® were added to the stock solution. Salinity was measured with a hydrometer to ±0.5 °/oo. Temperature was kept constant at 25°C. The test ranges for each species are given in Table 10. Both upper and lower salinity 96 hr TL_{50} values were determined.

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TABLE 10

RANGES OF TEMPERATURE AND SALINITY AND CONCENTRATION LEVELS OF TOXAPHENE TESTED FOR EACH SPECIES AND STAGE

Temperature		(°C)
Sesarma cinereum	all stages	25 - 40
Penaeus duorarum	all stages	25 - 40
Callinectes sapidus	S I - IV	25 - 40
Rhithropanopeus harrisii	all stages	25 - 40
Salinity		(°/oo)
Sesarma cinereum	S I	10 - 40
	S II, S III	5 - 45
	S IV	5 - 50
	Meg	0 - 55
Penaeus duorarum	Nauplius	20 - 50
	Protozoea	15 - 50
	Mysis	20 - 55
Callinectes sapidus	S I, S II	15 - 40
	S III	10 - 45
	S IV	15 - 45
Rhithropanopeus harrisii	S I	0 - 40
	S II, S III	0 - 45
Toxaphene		(ppb)
Sesarma cinereum	S I	0, 0.4, 0.5, 0.6
	S II	0, 0.01, 0.05, 0.1, 0.5, 1.0
	S III, S IV	0, 0.5, 1.0, 5.0, 10.0
	Meg	0, 5, 10

Toxaphene (Cont'd)

Penaeus duorarum	Nauplius, Mysis	0, 5, 10
	Protozoea	0, 0.5, 1.0, 5.0
Rhithropanopeus harrisii	S I	0, 10, 20, 30, 40, 50

Reduced Dissolved Oxygen Experiments

Four levels of dissolved oxygen were tested; 100% sat., 75% sat., 30% sat., and 10% sat. These oxygen levels were produced by passing regulated amounts of an air-nitrogen mixture over the culture dishes placed in large plastic bags. A beaker of water placed in each bag served as a measurement vessel. Oxygen levels in the measurement vessel were determined daily with an oxygen electrode (Galvanic Cell Oxygen Analyser, Precision Scientific Co.) which was calibrated by Winkler titration. Salinity and temperature were kept optimal for each test species.

Toxaphene Experiments

A stock solution with a concentration of 10 ppm in distilled water was prepared using an emulsified concentrated preparation (available from Southern Mill Creek Products, South Miami, Florida). Specifications for the concentrate are given in Materials and Methods, Fish. Test solutions were prepared by adding the requisite amount of the stock solution to medium of the optimal salinity for each test species to produce the desired concentration. Concentration levels to be tested were determined in screening tests. All tests were run at the optimal temperature for the test species. Animals were introduced immediately after solutions were prepared. The levels tested for each species are given in Table 10.

Glassware used for these tests was segregated from that used in all other parts of the study. These tests were conducted in a separate independent laboratory module to avoid any possibility of contamination.

Upon completion of the bioassay studies reported herein, a contract modification was negotiated to test the concentration of Toxaphene in simulated bioassay tests without animals. Three concentrations were prepared in the same manner as for bioassay tests in 1 liter Pyrex® culture bowls. The larger bowl size was necessitated by the sample volume required for analysis. A separate bowl was prepared for each sampling time. Analyses were made 3 hr., 48 hr. and 96 hr. after preparation of the solutions. The samples were extracted with hexane and the extract analyzed by gas chromatographic methods.

Since the concentration of Toxaphene recovered was less than expected, a subsequent test was conducted to develop a Toxaphene budget to account for all Toxaphene as one concentration level. The solution was prepared

in the usual manner. The container was a larger volume glass vessel to preclude possible errors in preparation of replicate solutions. Samples were taken in the same manner as described in the previous section concerning tests on fish species.

Interaction Tests

Five combinations of factors were tested: temperature-reduced dissolved oxygen, salinity-reduced dissolved oxygen, temperature-Toxaphene, salinity-Toxaphene, and reduced dissolved oxygen-Toxaphene. Conditions were established and maintained in the same manner as described above for the acute tolerance tests.

Levels for each parameter were selected to be sublethal on the basis of the acute tolerance tests, ranging from the optimal level to just below the 96 hr TL_{50} level. Each experiment was conducted at two (in a few cases three) levels of each parameter with all possible combinations.

In most tests there were two replicates of 20 animals each or a total of 40 animals at each test level. In a few tests, four replicates of 10 animals each were used. In tests with Penaeus duorarum, some experiments involved four replicates of 20 animals each for a total of 80 animals.

Specimens for histopathological study were preserved in 7% formalin in tap water with calcium carbonate buffer. The sections were stained with eosin-hematoxylin, a general stain. Selected slides were then examined for histopathological abnormalities. Control animals served as a basis for morphology.

Data Analysis

TL_{50} values after 96 hr. were determined in the acute tolerance tests by the graphical method of Doudoroff, et al. (1951, Anon., 1960) with the data for replicates pooled.

The data for replicates within each experiment were tested for homogeneity with the χ^2 test. The data for replicates was then pooled and the differences between test conditions evaluated by χ^2 tests. The data for control conditions is the best available data concerning the survival rates one can expect under optimal conditions for these animals which have a natural mortality greater than zero under natural conditions.

The mortality rates of all stages of each species were compared with a χ^2 test. Interspecific comparisons were not attempted because the numbers of stages of the several species varied. If this had been attempted the number of comparisons would have been astronomical.

The results of each interaction test was subjected to a 2-way classification analysis of variance test to define significant interaction effects. This test determines whether there is a statistically signifi-

cant differences between the sum of the effects for each variable acting alone and the effect of the variables acting simultaneously. The percent mortality data was transformed by the $\sin^{-1}\sqrt{M/100}$ function as suggested by Bartlett (1957) for probabilities or proportions with binominal variance. Percent mortalities of 0 and 100 were transformed by the method described in Snedecor with Cochran (1968) to correct for bias at extreme probabilities.

Synergism is defined here as an excess of mortality when the variables are acting together versus when acting alone, i.e. the effect is more than additive.

RESEARCH RESULTS

There is considerable variability in the number of deaths per day under given conditions. This variability is infrequently significant at the 95% level when tested by χ^2-tests except in Penaeus. In most cases where significant differences were obtained, these differences were significant on one day only, indicating differences in timing of death rather than different responses. This reflects the problems of infrequent observations (relative to the duration of the test) and small sample size. In the case of Penaeus, under extreme conditions in certain tests, nauplii and protozoea showed significant differences in mortality between replicates that cannot be discounted as a product of observation procedure and sample size. In summary, there are only a few cases where replicates differed in response to a given set of conditions and these may be examples of the 1 in 20 chance that a difference will occur within a single population. Therefore, the data for replicates has been pooled before analysis for differences in mortality between different test conditions.

The 96 hr temperature TL_{50} for all stages of S. cinereum ranged from 36.3 to 37°C. For stage II and Meg there was no significant difference between the mortality rate at 25 and 35 C although the rate of mortality was always higher at 35 C than at 25 C. The lower salinity TL_{50} for 96 hr ranged from 7.3 - 12.8 °/oo for all zoeal stages and was less than 1 °/oo for the megalopa, while the upper salinity TL_{50} for 96 hr ranged from 36.5 to 52.0 °/oo (including megalopa). There was no consistent trend in lower salinity 96 hr TL_{50} but the upper salinity 96 hr TL_{50} increased during development from stage I zoea to megalopa. The range of salinity over which no significant difference in mortality occurred was 15 to 35 °/oo for zoea I, 10 to 40 °/oo for zoea II and III and 5 to 50 °/oo for the megalopa. For stage IV all comparisons were significant, but the range of acceptable salinities for high survival is 10 to 40 °/oo. Thus while there was no uniform trend in 96 hr TL_{50} with advancing development, there was an increase in the optimal salinity range. The 96 hr TL_{50} for reduced dissolved oxygen concentration ranged from 49.0 to 57.5% saturation. These values are of low precision because the test interval (30% sat. to 75% sat.) was rather broad. There was no significant difference between the mortality rates at 75 and 100% saturation except in stage II. At 30% saturation mortality was total after 24 hours in stages I and IV, after 48 hours in stage III, after 96 hours for the megalopa. Only two zoeae survived after 96 hours at 30% saturation in stage II. The 96 hr Toxaphene TL_{50} was 0.054 ppb for stage I zoea, increased about tenfold to 0.76 ppb for stage II zoea and 0.74 ppb for stage III zoea and increased about tenfold again to 6.8 ppb for stage IV zoea and 8.4 ppb for the megalopa. Percent survival versus concentration at 96 hr is shown for each stage tested in Figure 1. In stage I there was no significant difference in mortality at all concentrations from 0.00 to 0.05 ppb, in stages II and III (significant on day 3 only) from 0.0 to 0.5 ppb and in stage IV and megalopa, from 0.0 to 5.0 ppb. However there was in every case higher mortality in the presence of Toxaphene than in the control. The 96 hr TL_{50} values are summarized in Table 11.

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The temperature-reduced dissolved oxygen interaction test showed no significant interaction effect for stage I zoeae or megalopae. For stage II zoea, percent mortality increased markedly at the highest temperature (34 C), but there was no effect of reducing the oxygen concentration. The difference in mortality at 34 C in the acute temperature test and the interaction test is attributed to the fact that the larvae were derived from different females in the two tests. For stages III and IV, there was no significant synergistic effect although the mortality when both parameters were extreme was higher than expected. In salinity-reduced dissolved oxygen tests there were no significant differences at any condition for any larval stage. In stage III, IV and megalopa there was a higher mortality when both parameters were extreme than was expected. In the megalopa there was an effect of salinity but not reduced dissolved oxygen. The data for interaction tests are summarized in Table 12.

TABLE 11.

TL_{50} VALUES AT 96 HR FOR EACH STAGE OF Sesarma cinereum

Stage	Temperature (°C)	Lower Salinity (°/oo)	Higher Salinity (°/oo)	Reduced Dissolved Oxygen (% sat.)	Toxaphene (ppb)
I	37.0	12.8	36.5	55.0	0.054
II	37.0	7.9	42.0	50.5	0.760
III	36.8	7.3	41.8	52.3	0.740
IV	36.3	8.7	46.0	49.0	6.8
Meg.	37.0		52.0	57.5	8.4

The temperature-Toxaphene tests show a significant synergistic effect for zoeal stages I and IV. The results for megalopae are anomalous, showing better survival when Toxaphene is present than in the controls. The difference is statistically significant at the 95% confidence level. This test should be repeated. The salinity-Toxaphene interaction tests also show a striking synergistic effect of high salinity and Toxaphene for stage I, with trends suggesting synergism in stages III, IV, and megalopa. For stage II there was no significant difference in mortality between any conditions in the test. In reduced dissolved oxygen-Toxaphene tests there was a significant synergistic effect in stages II and IV. The results for

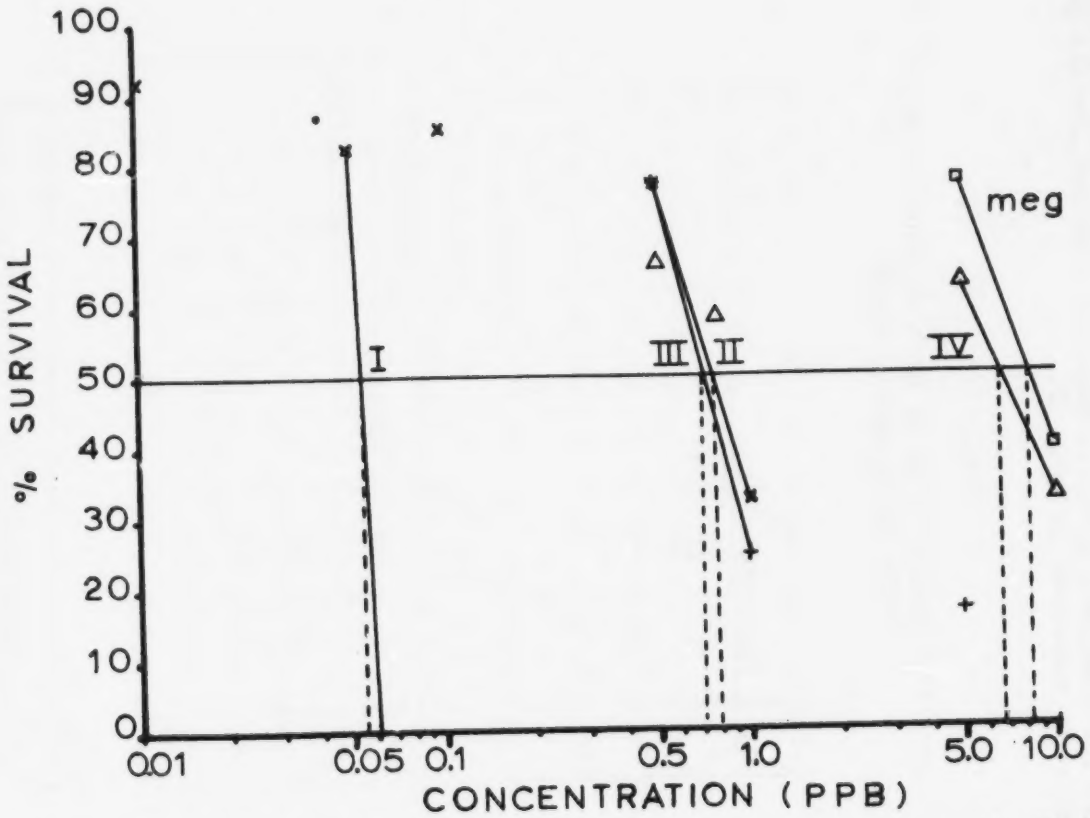

Figure 1. Percent survival versus Toxaphene concentration after 96 hr. exposure for the five zoeal and one megalopal stages of Sesarma cinereum.

· Stage I
x Stage II
+ Stage III
△ Stage IV
□ Megalopa

megalopae are anomalous. In this case mortality was significantly greater at 100% saturation than 75% saturation regardless of Toxaphene concentration which suggests an antagonistic effect. Further, mortality was significantly higher when Toxaphene was absent than at a Toxaphene concentration of 5 ppb. This result may be due to an error in data collection and the experiment should be repeated. The data for all interaction tests are summarized in Table 12.

TABLE 12.

PERCENT MORTALITY AFTER 96 HOURS IN INTERACTION TESTS FOR Sesarma cinereum

Stage I

		Temperature (°C)			Salinity (°/oo)		
		25	31	34	25	31	34
Reduced Dissolved Oxygen (% sat.)	75	1.25	6.75	6.25	2.50	6.25	8.75
	100	3.75	6.75	2.50	2.50	5.00	5.00

		Temperature (°C)		Salinity (°/oo)		Reduced Dissolved Oxygen (% sat.)	
		25	35	25	34	75	100
Toxaphene (ppb)	0.0	0.0	10.0	10.0	27.5	0	0
	0.05	0.0	62.5	17.5	100.0	5	0

Stage II

		Temperature (°C)			Salinity (°/oo)	
		25	31	34	30	40
Reduced Dissolved Oxygen (% sat.)	75	25.0	16.25	38.75	5.0	17.5
	100	20.75	13.75	35.00	10.0	20.0

TABLE 12 (continued)

		Temperature (°C)		Salinity (°/oo)		Reduced Dissolved Oxygen (% sat.)	
		25	35	30	40	75	100
Toxaphene (ppb)	0.0	2.5	22.5	7.5	0.0	20.0	2.5
	0.5	15.0	70.0	20.0	15.0	87.5	22.5

Stage III

		Temperature (°C)		Salinity (°/oo)	
		25	35	30	40
Reduced Dissolved Oxygen (% sat.)	75	25.0	77.5	10.0	57.5
	100	10.0	30.0	7.5	25.7

		Temperature (°C)		Salinity (°/oo)		Reduced Dissolved Oxygen (% sat.)	
		25	35	30	40	75	100
Toxaphene (ppb)	0.0	2.50	40.00	22.5	32.5	27.5	22.5
	0.5	2.50	67.75	22.5	45.0	32.5	10.0

TABLE 12 (continued)

Stage IV

		Temperature (°C)		Salinity (°/oo)	
		25	35	30	40
Reduced Dissolved Oxygen (% sat.)	75	32.5	77.5	35.0	47.5
	100	17.5	30.0	37.5	22.5

		Temperature (°C)		Salinity (°/oo)		Reduced Dissolved Oxygen (% sat.)	
		25	35	30	45	75	100
Toxaphene (ppb)	0	35.0	47.5	27.5	37.5	32.5	22.5
	5	27.5	80.0	40.0	85.0	100.0	32.5

Megalopa

		Temperature (°C)		Salinity (°/oo)	
		25	35	30	50
Reduced Dissolved Oxygen (% sat.)	75	15	30	12.5	47.5
	100	10	30	17.5	45.0

		Temperature (°C)		Salinity (°/oo)		Reduced Dissolved Oxygen (% sat.)	
		25	35	30	50	75	100
Toxaphene	0	17.5	17.5	10.0	40.0	7.5	25.0
	5	2.5	10.0	17.5	77.5	5.0	15.0

Interstage comparisons for S. cinereum revealed that there was a significantly different response to salinity and temperature for stage I versus all other stages and for stages III, IV and megalopa. The response to Toxaphene was significantly different for stage I versus all stages and stages II and III versus stage IV and megalopa.

The 96 hr temperature TL_{50} for P. duorarum was 36.3 - 36.5°C for nauplíar, protozoeal and mysis stages. There was no significant difference in mortality between 25 and 35°C in the mysis. The lower salinity TL_{50} at 96 hr was 19.5 - 23.5 °/oo, the upper salinity TL_{50} at 96 hr was 47.0 - 51.0 °/oo with a distinct increase from protozoea to mysis. There was no significant difference in mortality from 25 to 45 °/oo for the nauplius, from slightly above 20 to 45 °/oo for the protozoea, and from 25 to 50 °/oo for the mysis. The 96 hr reduced dissolved oxygen TL_{50} was 43.5 - 54.8% saturation. There was no significant difference in mortality between 75 and 100% saturation. Mortality at 30% saturation was not total but always greater than 75%. The 96 hr Toxaphene TL_{50} decreased from 2.2 ppb for nauplii to 1.4 ppb for mysis. Percent survival versus concentration at 96 hr is shown in Figure 2 for each stage tested. There was a significant difference in mortality between controls and experimental animals at all Toxaphene concentrations. These TL_{50} values are summarized in Table 13.

TABLE 13.

TL_{50} VALUES AT 96 HR FOR THE LARVAL STAGES OF Penaeus duorarum

Stage	Temperature (°C)	Lower Salinity (°/oo)	Upper Salinity (°/oo)	Reduced Dissolved Oxygen (% sat.)	Toxaphene (ppb)
nauplius	36.5	22.8	47.0	48.0	2.2
protozoea	36.3	19.5	47.3	43.5	1.8
mysis	36.5	23.5	51.0	54.8	1.4

The temperature-reduced dissolved oxygen test showed distinct synergistic effects in the mysis, and no synergism in the other stages. Significant interaction of salinity and reduced dissolved oxygen was not observed; however there was an increase in mortality at high salinity-reduced dissolved oxygen in every stage.

There was a significant interaction at every stage in temperature-Toxaphene tests. A significant synergism of salinity and Toxaphene was ob-

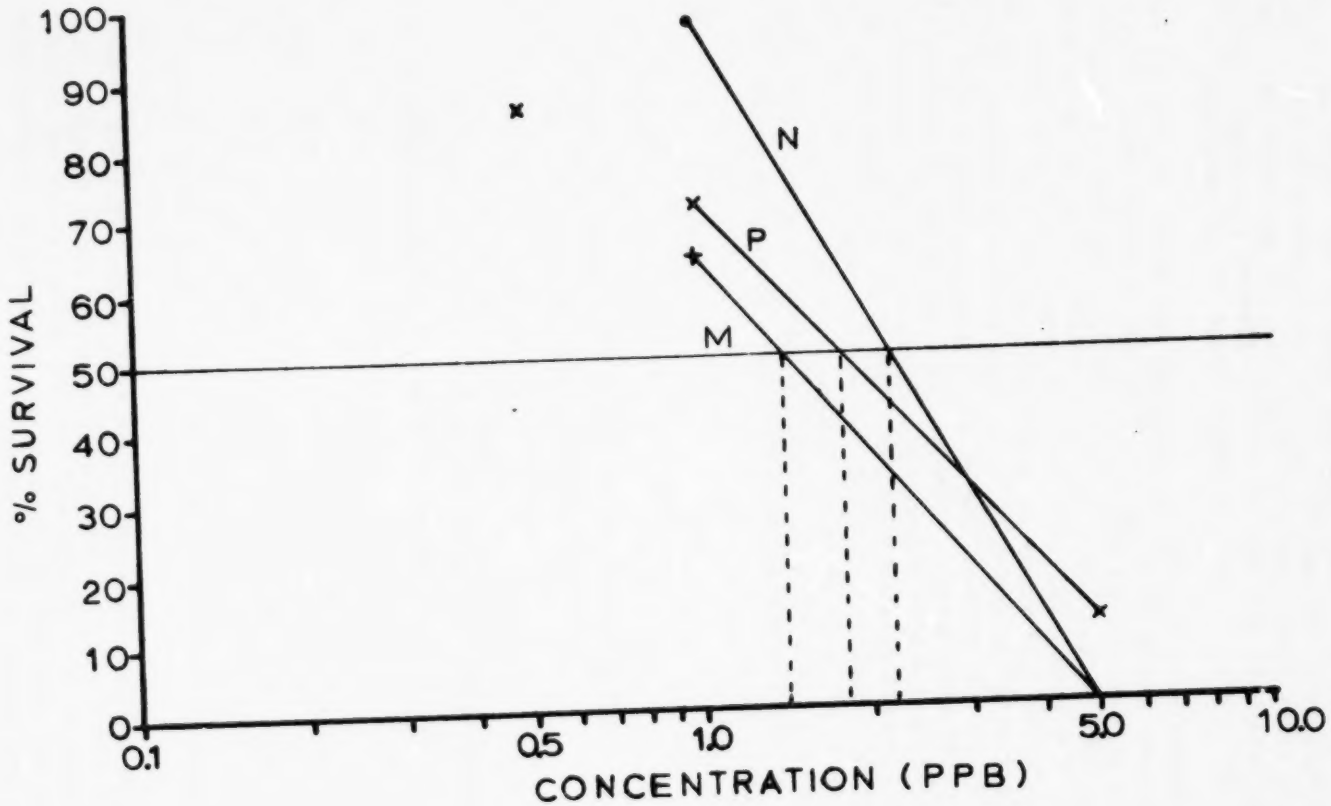

Figure 2. Percent survival versus Toxaphene concentration after 96 hr. exposure for the three larval stages of Penaeus duorarum.

o nauplii x protozoeae + mysis

served in the mysis stage. The nauplius stage showed significant mortality only when Toxaphene was present. At reduced oxygen levels a synergistic effect was not observed. The data for interaction tests are summarized in Table 14.

TABLE 14.

PERCENT MORTALITY AFTER 96 HOURS IN INTERACTION TESTS FOR Penaeus duorarum

nauplius

		Temperature (°C)		Salinity (°/oo)	
		25	35	35	45
Reduced Dissolved Oxygen (% sat.)	75	0.0	77.5	7.5	15.0
	100	5.0	42.5	0.0	10.0

		Temperature (°C)		Salinity (°/oo)		Reduced Dissolved Oxygen (% sat.)	
		25	35	35	45	75	100
Toxaphene (ppb)	0.0	10.0	32.5	0.0	0.0	0.0	0.0
	1.0	20.0	100.0	17.5	15.0	2.5	5.0

protozoea

		Temperature (°C)		Salinity (°/oo)	
		25	35	35	45
Reduced Dissolved Oxygen (% sat.)	75	30.0	40.0	12.5	25.0
	100	7.5	32.5	12.5	17.5

TABLE 14 (continued)

		Temperature (°C)		Salinity (°/oo)		Reduced Dissolved Oxygen (% sat.)	
		25	35	35	45	75	100
Toxaphene (ppb)	0.0	5.0	37.5	22.5	25.0	32.5	10.0
	1.0	27.5	97.5	22.5	40.0	87.5	15.0

Mysis

		Temperature (°C)		Salinity (°/oo)	
		25	35	35	50
Reduced Dissolved Oxygen (% sat.)	75	37.5	70.0	37.5	67.5
	100	35.0	37.5	32.5	42.5

		Temperature (°C)		Salinity (°/oo)		Reduced Dissolved Oxygen (% sat.)	
		25	35	35	50	75	100
Toxaphene (ppb)	0	15	25	17.5	32.5	12.5	15.0
	1	30	90	15.0	67.5	12.5	15.0

In interstage comparisons for P. duorarum, the responses of all stages to all parameters differed significantly even though 96 hr TL_{50} values were quite similar. This resulted from low survival under all conditions including optimal for the mysis stages, relative to the other stages and differences in the shape of the mortality curves for each stage.

Acute tests for C. sapidus were completed for stages I to IV for environmental parameters only. This resulted from problems encountered in obtaining ovigerous females and low survival rates of early stages. Low

survival rates of early larval stages are believed to be representative of survival rates in nature and not solely a reflection of inadequacies of culture technique.

The 96 hr temperature TL_{50} was 35.7 to 37°C for the first four zoeal stages of C. sapidus. There was no significant difference in mortality from 25 to 35 C except in Stage III. The lower salinity TL_{50} at 96 hr was 17.5 to 19.0 °/oo for stages I, II, and IV, but only 14.0 °/oo for stage III. This latter value is lower than expected. The upper salinity TL_{50} at 96 hr ranged from 37.0 °/oo for stage I and II zoeae to 41 to 42 °/oo for stage III and IV zoeae respectively. There was no significant difference in mortality from 20 to 35 °/oo for stage I and II, from 20 to 40 °/oo for stage III and from slightly above 30 to 40 °/oo for stage IV. The 96 hr reduced dissolved oxygen 96 hr TL_{50} was 56.0 to 60.5% saturation for all stages tested. There was no significant difference in mortality between 75 and 100% saturation except in stage I. The 96 hr TL_{50} values are summarized in Table 15.

TABLE 15.

TL_{50} VALUES AT 96 HR FOR THE FIRST FOUR ZOEAL STAGES OF Callinectes sapidus

Stage	Temperature (°C)	Lower Salinity (°/oo)	Higher Salinity (°/oo)	Reduced Dissolved Oxygen (% sat.)
I	37.0	17.5	37.0	56.0
II	36.0	18.3	37.0	60.5
III	35.7	14.0	41.0	59.0
IV	36.3	19.0	42.0	60.5

Interaction tests were completed for stage I through III zoea (excluding pairs involving Toxaphene). For stage I and II zoeae there was a synergistic effect between elevated temperature and reduced oxygen levels. For stage III, there was an effect noted only for temperature. In salinity-reduced dissolved oxygen tests, there was a synergistic effect in stage I but only an effect of salinity in stage III. The data for interaction tests are summarized in Table 16.

TABLE 16.

PERCENT MORTALITY AFTER 96 HOURS IN INTERACTION TESTS FOR THE FIRST THREE ZOEAL STAGES OF Callinectes sapidus

Stage I

		Temperature (°C)		Salinity (°/oo)	
		25	35	25	35
Reduced Dissolved Oxygen (% sat.)	100	7.5	35.0	5.0	12.5
	75	25.0	100.0	27.5	67.5

Stage II

		Temperature (°C)		Salinity (°/oo)	
		25	35	30	35
Reduced Dissolved Oxygen (% sat.)	100	20.0	25.0	25	35
	75	27.5	77.5	30	60

Stage III

		Temperature (°C)		Salinity (°/oo)	
		25	35	30	40
Reduced Dissolved Oxygen (% sat.)	100	25.0	62.5	22.5	40.0
	75	25.0	65.0	37.5	55.0

In interstage comparisons for C. sapidus, the response to salinity differed between all four stages tested. The response to temperature was the same for stages I and III. The response of stage IV to reduced dissolved oxygen was significantly different from the other stages.

Ovigerous R. harrisii were scarce during the course of this study, perhaps as a result of the bi-annual severe drought in southern Florida. Temperature tests were completed for all stages. The 96 hr temperature TL_{50} was 37.0 to 37.3°C in every case. There was no significant difference in mortality between 25 and 35°C except in stage III. Salinity tests were completed only for stages I to III. The lower salinity TL_{50} at 96 hr does not exist for stage I zoea and lies somewhere between 0 and 5 °/oo for stages II and III. Insufficient test concentrations in this salinity range preclude a more exact determination of the lower salinity 96 hr TL_{50}. The 96 hr upper salinity TL_{50} was 37 °/oo for stage I, 47.5 °/oo for stage II and 42.5 °/oo for stage III. There was no significant difference in mortality between 0 and 35 °/oo in stage I and between 5 and slightly less than 40 °/oo in stages II and III. The 96 hr reduced dissolved oxygen TL_{50} was 45% for stage I, and 41.0% for stage II, the only stages tested. There was no significant difference in mortality between 75 and 100% saturation for stage I, but significant differences were found between 30 and 75% saturation and 10 and 30% saturation for both stages. The 96 hr Toxaphene TL_{50} for stage I was 43.75 ppm with no other tests completed (Figure 3). There was no significant difference in mortality over the range 0.0 to 40.0 ppb Toxaphene. The 96 hr TL_{50} values are summarized in Table 17.

TABLE 17.

TL_{50} VALUES AT 96 HR FOR EACH STAGE OF Rhithropanopeus harrisii

Stage	Temperature (°C)	Lower Salinity (°/oo)	Upper Salinity (°/oo)	Reduced Dissolved Oxygen (% sat.)	Toxaphene (ppb)
I	37.0	does not exist	37.0	45	43.75
II	37.3	(2.5)	47.5	41.0	288-345*
III	37.0	(2.5)	42.5		290-430*
IV	37.0	does not exist	35		
Meg	37.0				

*Based on preliminary screening tests

The only interaction test completed was salinity-reduced dissolved oxygen for stage I. There was no evidence of combined action of these parameters. These data are summarized in Table 18. Survival was signi-

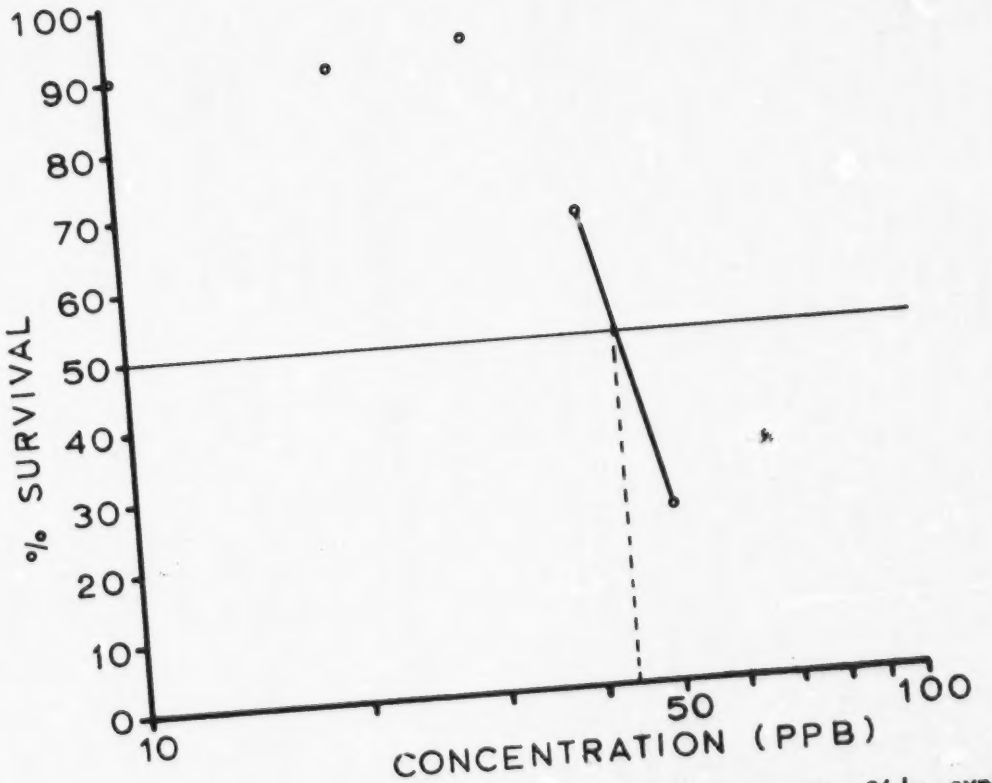

Figure 3. Percent survival versus Toxaphene concentration after 96 hr. exposure for zoeal stage I of Rhithropanopeus harrisii.

ficantly better at 34 °/oo, 100% saturation than at any other salinity and at the same salinity in the acute test for salinity.

TABLE 18.

PERCENT MORTALITY AFTER 96 HOURS IN AN INTERACTION TEST FOR Rhithropanopeus harrisii

		Salinity (°/oo)		
		25	31	34
Reduced Dissolved Oxygen (% sat.)	75	10.00	7.50	11.25
	100	6.25	3.75	0.00

Some preliminary screening tests were completed in addition to the above tests. A preliminary test of the effect of salinity on stage IV zoeae showed that the lower salinity 96 hr TL_{50} does not exist. The salinity range tested did not include the upper salinity 96 hr TL_{50} which exceeds 35 °/oo. Two preliminary tests of Toxaphene on stage II zoea give 96 hr TL_{50} values of 288 ppb and 345 ppb. These tests are summarized in Table 17.

In interstage comparisons for R. harrisii, temperature is the only parameter for which a complete series of comparisons is possible. There was no significant difference in the response to temperature for any stage.

The data for the analysis of Toxaphene over time in simulated bioassay tests are presented in Table 19. The concentration at 3 hr was approximately 29.2% of the calculated value at a nominal concentration of 0.5 ppm and 89.6% at 0.1 ppm. In the other concentration (0.05 ppm) the assayed concentration exceeded the expected by about 100%. The reason for this aberrant result is not apparent.

In a separate experiment we determined the distribution of Toxaphene in the test system after 24 hr at a concentration of 0.50 ppm. The data is presented in Table 20. At 3 hr the Toxaphene concentration was 0.359 ppm, and at 24 hr it was 0.475 ppm. This increase in concentration at 24 hr was noted in several duplicate experiments to the one reported here. The explanation seems to be that the Toxaphene went into solution slowly. The previous test indicates that the concentration decreased to a level

TABLE 19.

ASSAYED TOXAPHENE CONCENTRATION IN SIMULATED BIOASSAY TEST WITH GLASS CULTURE VESSELS

Nominal Concentration (ppm)	Calculated Concentration (ppm)	Assayed Concentration (ppm) Days 0	2	4
0.50	0.479	0.140	0.130	0.058
0.10	0.096	0.086	0.075	0.078
0.05	0.048	0.099	0.210	0.084

approximately equal to that at 3 hr, most probably by adsorption to the glass. At 24 hr, 13 µg were present in particulate form, and 10 µg were adsorbed to the glass. No absorbed Toxaphene was observed. The total Toxaphene recovered was 892.1 µg or 93.1% of the amount introduced. Considering the number of assays involved with their attendant errors, and the crude nature of the procedure to estimate the particulate fraction, this percent recover is very complete.

TABLE 20.

TOXAPHENE BUDGET AT 24 HR IN SIMULATED BIOASSAY TEST WITH GLASS CULTURE VESSELS

Fraction	µg
soluble	869.1
particulate	13.0
adsorbed	10.0
absorbed	0.0
TOTAL	892.1

ug Toxaphene introduced	958 µg
% recovered	93.1 %

The lack of conformity between the measured concentration at 3 hr in the concentration-time study and the Toxaphene budget test at a nominal concentration of 0.5 ppm is very puzzling. The anomalous results in the concentration-time study may provide an explanation since the percentage in solution at this nominal concentration was surprisingly low. We place greater confidence in the second test because the data was repeatable in replicate tests. The lack of conformity may also derive from the use of one liter culture bowls in the concentration time study versus larger volume glass containers with a different configuration and hence surface to volume ratio in the budget test.

TABLE 21.

SUMMARY OF CRUSTACEAN SPECIMENS
EXAMINED FOR HISTOPATHOLOGICAL STUDY

Species	Stage	Number of Control Animals Examined	Number of Experimental Animals Examined	Toxaphene Concentration (ppb)
Sesarma cinereum	I	3	2	0.03
			2	0.04
	II	6	10	0.04
	III	6	5	0.04
Callinectes sapidus	I	3	1	0.0005
			1	0.00001
	II	7	6	0.01
Rhithropanopeus harrisii	I	8	3	10.0
	II	7	2	100.0
			2	200.0
	III	10	2	400.0
	IV	10	3	200.0

Histopathology

Table 21 specifies the species, stage, number of animals, and exposure levels for those slides examined. In stage I of S. cinereum, the hepatopancreas had vacuolated granulated cells in one specimen coupled with contraction of the intestine. The other specimens showed no noticeable effects. In stage II, 20% of the specimens showed no effects, 50% had vacuolated granulated hepatopancreas cells, and 30% exhibited

necrotic spaces. Four of these specimens exhibited intestinal contraction, one a slight dilation, and five no apparent effect. In stage III, all specimens exhibited extensive degeneration of the hepatopancreas and 80% showed contraction of the intestine.

Stage I C. sapidus larvae showed no histological changes compared to controls at either 5×10^{-4} or 1×10^{-5} ppb Toxaphene. In stage II, hepatopancreas damage similar to that found in S. cinereum was noted in 50% of the specimens. The exposure concentration for stage II was 1×10^{-2} ppb, several orders of magnitude higher than the exposure level for stage I.

The same histological effects were observed in stage I, II, and IV R. harrisii larvae. In stage I, one specimen also exhibited necrosis of the intestine and sloughing of the stomach epithelium. Stages II and IV exhibited damage to the nervous tissue with irregular spaces apparent between the fibers.

In all three species, deposition of orange-brown pigment has been noted in body spaces, especially along the intestine. This pigment deposition is not observed in control animals. The pigment is not soluble in water, alcohol, or xylene.

No histological changes were observed in other organs of the zoeae. There was no evidence of bacterial or fungal invasion in the Toxaphene exposed animals.

DISCUSSION

The 96 hr temperature TL_{50} for all stages of all species tested ranged from 35.7 to 37°C. This range is comparable to upper lethal temperature limits for a variety of warm acclimated organisms (see for example Prosser, 1961). In the experiments described here the acclimation temperature was in every case 25°C, or about the optimal temperature for each species. No data are available in the literature on the upper lethal limits for animals acclimated at lower or higher temperatures. One would expect the values for those stages of Callinectes sapidus not tested to fall within the above range.

Costlow, Bookhout, and Monroe (1960) obtained complete development of S. cinereum at 30°C, 20 and 25 °/oo, but not at salinities above and below this range. Even at these salinities, the percent reaching the juvenile stage at 30°C was less than 10 percent. A similar result was obtained for R. harrisii though the range of salinities permitting complete development and the level of success, were both much greater (Costlow, Bookhout and Monroe, 1966). This is not surprising in this very eurytolerant species.

The effect of temperatures on larvae of P. duorarum has recently been studied by Thorhaug, Devany, and Murphy (1970). Development of nauplii to protozoea occurred only from 24 to 31.5 C although survival was high for a 10 hour period from 15 to 37°C. The median lethal limit for protozoea I and III was 37.0 to 37.8 C and 35.7 to 36.7 C respectively which agrees favorably with our results although their tests were of short duration (equal to the intermolt duration of each stage, generally 18 to 24 hours). The upper lethal limit for the mysis stage was 36.9 to 37.4 C, again very similar to our result.

The species tested differ markedly with respect to the salinity range in their respective habitats. S. cinereum is an estuarine species which spends a major portion of its life above the waterline though near salt water. However, like other semi-terrestrial crabs, it must return to water for reproduction (Williams, 1965). The larvae have a lower 96 hr TL_{50} ranging around 10 °/oo which is near the level at the upstream limit of natural distribution of adults. The upper salinity limit increased from 36.5 °/oo to 52 °/oo from stage I zoea to the megalopa. Larvae flushed from an estuary into the sea would therefore be capable of surviving and developing and could reinvade the estuary as megalopae or juveniles. Complete development to the juvenile for S. cinereum has been observed at 20 and 26.5 °/oo but not at 12.5 and 31.1 °/oo at 20, 25 and 30°C (Costlow, Bookhout, and Monroe, 1960).

The life cycle of P. duorarum has been reviewed in Williams (1965). Early juveniles migrate into estuaries where growth is rapid. The adults migrate out of the estuary and spawn in open waters. The larval stages are passed in the lower reaches of the estuary or offshore. The lower salinity limit was 19.5 °/oo or above, commensurate with the normal dis-

tribution of these larvae. The upper salinity limit was very high at ca 50 °/oo, far in excess of concentrations apt to be encountered in nature.

C. sapidus migrates upstream during the juvenile period and grows to adult size in brackish or fresh waters. When sexual maturity is reached, it migrates in the reverse direction into regions where salinities exceed 20 °/oo where eggs hatch (Van Engel, 1958). It has previously been demonstrated that eggs of C. sapidus do not hatch at salinities lower than about 15 °/oo and complete development only occurs at salinities above 20 °/oo (Sandoz and Rogers, 1944; Costlow and Bookhout, 1959). This salinity is somewhat higher than the lower lethal limit based on a TL_{50} at 96 hr of 17.5 to 19.0 °/oo (and only 14.0 °/oo for stage III) reported here. However, it was not determined as a property of the contract research whether larvae which survived after 96 hours could complete development to the juvenile at these low salinities. The upper salinity limits of 37 to 42 °/oo are slightly higher than normal seawater and probably exceed levels ever encountered by larvae in nature.

Costlow (1967) investigated the effects of temperature and salinity on the development of the megalopa of C. sapidus. The megalopa developed to the juvenile over the range of 15 to 30°C at suitable salinities and over a range of 5 to 40 °/oo at suitable temperatures. Development at 5 °/oo occurred only at 25 and 30°C and at 40 °/oo at all temperatures tested. No data is available from this study for comparison.

R. harrisii is a brackish water crab which as an adult ranges from virtually fresh water to mid salinity regions, although it is capable of surviving in normal seawater. For stages I to IV zoea, the lower salinity limit either does not exist or is well below 5 °/oo. The upper salinity limit was 37 °/oo or above for all zoeal instars. Costlow, Bookhout and Monroe (1966) observed complete development for 8% of the larvae cultured at 25°C, 40 °/oo, and 22% of the larvae cultured at 25°C, 35 °/oo.

It has been reported in numerous studies that death under adverse conditions is frequently associated closely with molting (see for example Roberts, 1971a). It would seem that when the larva molts, its defenses are lowered making it more susceptible to stress conditions. In these tests, the same effect was noted in cases where molting occurred during the course of the test. However, in the majority of the tests, molting did not occur during the course of the test.

Because of the above observation, one must consider whether TL_{50} determinations after 96 hr are meaningful for these organisms. The TL_{50} value at 96 hr would presumably be higher than is actually the "true" value, that is the animals appear more tolerant than they really are. Analysis of this point was not required by contract. The data of Costlow, et al. (1960, 1962, 1966) is based on a different approach and encompasses this consideration; however, direct statistical comparisons of their data with that presented here is not possible. The permissible

ranges for temperature and salinity derived from our 96 hr TL_{50} determinations are broader than those based on the ability of larvae to complete development. This supports the above contention.

The reduced dissolved oxygen TL_{50} at 96 hr was near 50% saturation for S. cinereum and P. duorarum which are moderately tolerant species with regard to many parameters. C. sapidus, the least tolerant species had a reduced dissolved oxygen 96 hr TL_{50} approaching 60% saturation, whereas the most tolerant species, R. harrisii, had a value 41-45% saturation for all stages tested. The trend of decreasing tolerance of larvae to reduced dissolved oxygen with increasing fragility of the larvae is quite reasonable. No data are available in the literature for comparison.

S. cinereum was very sensitive to Toxaphene in zoeal stage I, increased in tolerance by ten fold in stages II and III, and ten fold again in stages IV and megalopa. The same trend is suggested by the data for R. harrisii. However, R. harrisii was approximately 1000 times more tolerant than S. cinereum in stage I and about 400 times more tolerant in stages II and III. Complete data are not available for C. sapidus; however a few preliminary experiments suggest that this species is much less tolerant than S. cinereum. Values for P. duorarum in all stages were slightly higher than values for stage II and III S. cinereum. There was a slight decrease in tolerance as the larvae developed from the nauplius to the mysis stage.

In the temperature-reduced dissolved oxygen interaction tests, there was no synergistic effect for any stage of S. cinereum. For C. sapidus, however, there was a synergistic effect in stages I and II, though surprisingly, not in stage III. A synergistic effect of temperature-reduced dissolved oxygen was observed only in the mysis stage of P. duorarum.

The results for S. cinereum in salinity-reduced dissolved oxygen tests showed the same trend with no synergistic effects for any stage. For C. sapidus there were large synergistic effects for stage I only. The only data for R. harrisii showed no synergistic effect for stage I. There was no statistically significant evidence of synergistic action on all stages of P. duorarum.

Data for interaction tests involving Toxaphene are available for S. cinereum and P. duorarum. There was a significant interaction of temperature and Toxaphene for all stages of P. duorarum and for stages I and IV of S. cinereum. Similarly, there was a synergistic effect of elevated salinity for stage I of S. cinereum and the mysis stage of P. duorarum. There was also a synergistic effect of reduced dissolved oxygen for zoeal stages II and IV of S. cinereum. As noted above, the results for the megalopa are exactly the opposite. This antagonistic effect is suspect. It is suggested that this test be repeated. A synergistic effect of reduced dissolved oxygen was also noted for the mysis of P. duorarum but not earlier stages.

No tests were conducted on the interaction effects of temperature and salinity. Costlow, Bookhout and Monroe (1960, 1966) have shown that there are such effects for all stages of S. cinereum and R. harrisii. Costlow (1967) reported the same types of effects for the megalopa of C. sapidus.

The present results indicating a synergistic interaction between two test parameters could reflect one of three processes. The simultaneous action of two parameters may accelerate the rate at which the response is achieved with no change in the final degree of response (ultimate lethal dose). Alternatively, the simultaneous action of two parameters may lead to a change in the ultimate lethal dose which exceeds the sum of independent responses to each parameter with no change in the time to achieve the ultimate lethal dose. Finally, both rate and ultimate lethal dose may be affected. These alternatives cannot be distinguished from the present tests because the ultimate lethal dose cannot be estimated from the data. It is doubtful that the TL_{50} equals the ultimate lethal dose, especially since the most sensitive period (ecdysis) in the larval period generally does not occur within the test period.

The evidence presented here indicates that in establishing permissible levels of Toxaphene in natural environments, consideration must be given to the effects of environmental factors such as temperature, salinity, and oxygen concentration, especially at the extremes of the range tolerated by the species which will be exposed to the pesticide. On the basis of available data and the refractory nature of C. sapidus larvae in culture, one would expect this species to be least tolerant of these tested with significant synergistic effects.

The analyses of Toxaphene concentration indicate that 75 to 90% of the Toxaphene was in solution (excluding suspect assays). The concentration of Toxaphene in solution increased from 3 hr to 24 hr after solution preparation and then decreased slowly until the end of the test. This would indicate that TL_{50} estimates may be 10 to 25% higher than the actual TL_{50}'s. Correction of the TL_{50} values is not warranted as the basis of the available analysis data. These results point out the necessity of frequent chemical analyses with every bioassay test on the actual test solutions.

The only histological changes observed in preliminary studies for Toxaphene-exposed S. cinereum and C. sapidus were a contraction of the intestine and progressive vacuolation and necrosis of the hepatopancreas. These conditions may develop more rapidly in older than younger zoeae; however, an inadequate number of larvae have been examined to be certain on this point. The same conditions were observed with R. harrisii. In addition, R. harrisii showed some deterioration of the nervous system.

During exposure tests, it was observed that animals in high Toxaphene concentrations became disoriented, swam slower, and frequently flexed and extended the telson. This telson flip response is a common response

of decapod larvae to stressful conditions (Roberts, 1971b) and not a specific response to Toxaphene. There was no histological evidence of damage to the nervous system except in R. harrisii.

The evidence presently available suggests that the site of action of Toxaphene is the digestive system, in particular the hepatopancreas. It is desirable to examine additional specimens exposed for long periods to lower concentrations or specimens exposed to high concentrations for shorter times. In this way it would be possible to follow the course of hepatopancreas degeneration.

GENERAL DISCUSSION

The results of temperature tolerance tests for juvenile fishes and all decapod larvae were in close agreement (96 hr TL_{50} = 35 to 37°C). As noted above, this coincides with values for numerous other warm acclimated organisms (Prosser, 1961). In contrast, bass larvae were slightly less resistant (96 hr TL_{50} = 33.5°C) and bass embryos still less tolerant (96 hr TL_{50} = 31.5°C).

The heated effluent from fossil fuel plants frequently reaches levels of 32-33°C (and sometimes 39-40°C) near the outfall, and dissipates slowly with distance from the outfall. This temperature is within the tolerance range of the species tested (at least for temporary exposures) except for bass eggs and larvae. Effluents closer in temperature to the lethal limit can be expected to cause considerable mortality.

Harder (1952a, 1952b) demonstrated that species in several phyla including Arthropoda (decapods) and Chordata (fishes) are capable of responding to gradients of environmental parameters by avoidance. Most of his work related to avoidance of salinity gradients, but he did conduct a few experiments with temperature gradients. It is likely that species used in the present tests have this capability although no studies of this potentiality were required under the present contract. Such avoidance of waters of adverse thermal levels would serve to prevent high mortalities but at the same time would restrict the volume of water available to the species for support of its populations.

The salinity range tolerated by all species corresponds to the salinity ranges within their respective distribution ranges or, in some cases, slightly exceeds the latter range. This latter observation can be explained in several ways. First, it may reflect the failure to consider the most sensitive periods for the stages tested as noted in each of the proceeding sections. Second, it may result from the inability of the organisms to compete with other animals when living in waters with salinities approaching the lethal limits. Third, it has been demonstrated that some decapod larvae avoid water with a salinity within the range acceptable for complete development but approaching the lethal limit (Scarratt and Raine, 1967; Roberts, 1971b). It is reasonable to expect the same thing to be true for species tested under this contract.

While oxygen availability is a limiting factor only in local areas and then rarely, it may become limiting in regions of primary and secondary pollution where BOD and COD are high. Based on the studies reported here, concentrations below 50 percent saturation at optimal temperature and salinity are lethal for decapod larvae, larval and

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juvenile bass, and juvenile mullet. Bass embryos and juvenile pompano tolerate oxygen levels of 15.3% and 18% saturation respectively. There is a trend for species eurytolerant to other stresses to be slightly more tolerant of reduced oxygen concentrations than less eurytolerant species. There are significant synergistic effects with temperature and salinity which probably reflects the increased metabolic demands in meeting the challenge of extreme temperatures and salinities.

Avoidance of low oxygen concentrations has been tested in four species of fish by Whittmore, Warren, and Doudoroff (1960). Included in this study were late juvenile - early adult largemouth bass (Micropterus salmoides, 50-90 mm total length). This species avoided water with an oxygen concentration of 1.5 mg/l (15.8% saturation) but did not avoid concentrations of 3.0 mg/l (32.6% saturation) or above. Two other species tested (chinook salmon and coho salmon) exhibited avoidance at 4.5 mg/l (20.6% saturation) at 22.8°C and 3.0 mg/l (28.3% saturation) at 13.2°C. Bluegills responded in the same manner as largemouth bass. These authors concluded that these fishes avoided waters with oxygen concentrations higher than that concentration causing mortality within 24 hours. This point merits further investigation.

The fishes studied at all stages were much less tolerant of Toxaphene than either Sesarma cinereum, Rhithropanopeus harrisii or Penaeus duorarum. We suspect that Callinectes sapidus is much less tolerant than any other species tested. The degree to which fish are less tolerant than decapod larvae is much greater than indicated by direct comparison of TL_{50} values defined here because the values for fish are gross overestimates.

It has been shown that the sheepshead minnow (Cyprinodon variegatus) avoided water containing certain pesticides (Hansen, 1969) and salmon avoided heavy metal ions (Sprague, 1964). No similar studies have been reported in the literature for decapod larvae. It seems reasonable that some avoidance capability exists for the fish and decapods studied although tests of this hypothesis were not required under this contract. If this is true, it implies that habitat limitation might result from pesticide presence rather than increased mortality. Obviously this would not be possible for fish embryos and perhaps also sac-fry larvae. However, these stages are more tolerant than juveniles of largemouth bass.

Toxaphene has very low solubility in aqueous media. It enters the environment in a xylene carrier which is non-miscible with water. In preparing our test solutions a Toxaphene-xylene solution was used. The maximum amount of xylene which could have been present in the final test solutions was less than 5×10^{-8} ml/l at the highest Toxaphene concentration tested (50 ppb Toxaphene) assuming xylene remained in the aqueous solutions. No xylene control groups were tested. Xylene residues (and volatile hydrocarbons) evaporate rapidly and hence could have had little or no effect on our results. Better solution of Toxaphene would probably have been achieved by including a surfactant in solution preparation as suggested by Mount and Brungs (1966).

Although the plastic-lined vessels used to maintain fish have been demonstrated to have life support characteristics as good as or better than glass, they are not suitable for bioassay tests involving pesticides. Plastics rapidly ad- and absorb organic pesticides. Our tests suggest that 92% is sorbed within 24 hours and virtually 100% within 48 hours. As has already been pointed out the TL_{50} values obtained for fish are gross overestimates and hence not reported.

In many interaction tests, synergistic effects between Toxaphene and all environmental parameters were suggested by the data for crustaceans but in only a few cases were these effects statistically demonstrable. The failure to show statistical significance for decapod larvae is attributable to several factors: choice of test levels, number of replicates, number of test levels. More attention should be paid to statistical requirements to demonstrate synergism in experimental design.

ACKNOWLEDGEMENTS

Aquatic Sciences, Inc. gratefully acknowledges the following for valuable assistance in obtaining experimental animals for use in this project: John Anderson, U. S. Bureau of Sport Fisheries and Wildlife fish hatchery, Welaka, Florida; William Ashe, U. S. Bureau of Sport Fisheries and Wildlife fish hatchery, Frankfort, Kentucky; C. Yancey Byrd, III, Delray Beach, Florida; Roger M. Cooper, U. S. Bureau of Sport Fisheries and Wildlife fish hatchery, Yankton, South Dakota; Sheldon Deal, Riviera Beach, Florida; J. Walter Dineen, Central and Southern Florida Flood Control District, West Palm Beach, Florida; Peter Dingler, Miami, Florida; Fisher Wholesale Seafoods, Port Canaveral, Florida; O. Earl Frye, Jr., Director, Florida Game and Fresh Water Fish Commission, Tallahassee, Florida; Maj. Brantley Goodson, Chief of Law Enforcement, Florida Game and Fresh Water Fish Commission, Tallahassee, Florida; Robert M. Ingle, Florida Department of Natural Resources, Tallahassee, Florida; Marilyn Metzger, Port Salerno, Florida; Lt. Morgan, Titusville, Florida; Lt. Purdon, West Palm Beach, Florida; Vernon C. Ogilvie, Florida Game and Fresh Water Fish Commission, West Palm Beach, Florida; Larry Schafer, St. Georges Packing House, Ft. Myers Beach, Florida; Herman Summerlin, Ft. Pierce, Florida; David Sweat, Sea Farms Inc., Key West, Florida; John W. Woods, Chief of Fisheries, Florida Game and Fresh Water Fish Commission, Tallahassee, Florida. Robert Zensen of the Central and Southern Florida Flood Control District, West Palm Beach, Florida provided a permit and levee key allowing us to have access to water conservation areas for the purpose of collecting animals. The following people are acknowledged for their assistance in conducting experiments with fish: Karen Ellerie, Martin Hyatt, Edward Mistarka, Jackie Schroeder, Brad Stirn, Ron Weiss. The studies with decapod larvae were carried out through the assistance of: Margaret Cutts, Maura Haran, Gail Holland, Linda Kapsch, Mary Maynard, Dianne Rocheleau, Peggy Van Arman, Margaret van Montfrans, Mark Widmer, Cathy Wood. The collection of many specimens and responsibility for life support by Kent Norris, Buddy Payne, and Robert Rutter are gratefully acknowledged. Phyllis Graglia and Joel Van Arman are acknowledged for preparation of animals for histological examination. Teen Kline and Jeff Peterson assisted with photography. Mike Vaillant prepared the final drawings for this report and was responsible for routine chemical analysis. Dr. Al Smith kindly examined slides of decapod larvae for histopathological changes. Dr. Richard G. Domey gave advice on statistical questions and performed the statistical tests.

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GLOSSARY

acclimation: the compensatory change in an organism under maintained deviation of a single environmental factor.

acclimatization: the compensatory changes in an organism undergoing multiple natural deviations of milieu.

antagonism: the interaction effect of two (or more) toxicants which is less than the sum of the effect of each acting separately.

blastula: that embryonic stage containing a cavity, the blastocoel with an associated layer of cells, the blastoderm.

brackish water: referring to waters of a salinity between 0 and 10 °/oo.

epithelium: any sheet of cells which covers and lines free body surfaces, internal or external.

eury-: prefix meaning wide as in eurytolerant.

gastrula: that embryonic stage consisting of two tissue layers, ectoderm and entoderm.

gravid: refers to female animals possessing ripe eggs.

hepatopancreas: the organ of the digestive system of crabs producing digestive enzymes; also called "liver".

histopathology: the study of tissues and disease.

interaction effect: effects produced by two factors acting on an organism together (see antagonism and synergism).

juvenile: that stage in the life history when an animal has adult morphology but is not sexually mature.

larva (e): that stage in the life history transitional from egg to adult. It is an embryonic stage. In present usage, it refers only to free-living forms.

megalopa (e): the last larval stage of brachyuran and anomuran decapods possessing larval and juvenile characteristics.

melanophore: pigment-bearing and pigment-producing cells having the black pigment melanin.

metabolite: any of a variety of biologically active compounds produced by enzymatic reactions.

molt: the process of shedding the exoskeleton necessary for growth in anthropods including decapods.

mysis (es): that larval stage in the development of penaeids in which propulsion is by thoracic appendages and the abdominal appendages appear (corresponds to the zoeae of other decapods in part).

nauplius (i): the larval stage in the development of penaeids in which propulsion is by the antenual appendages. Only the antennules, antennae, and mandibles are functional.

necrosis: localized death and disintegration of tissue.

neurula: that embryonic stage in which organ systems are differentiated; characterized by neural tube formation.

notochord: a long, flexible, rodlike structure found in all vertebrate embryos.

organogenesis: the process of organ formation.

ovigerous: refers to female crabs bearing developing eggs on the abdominal appendages.

pelagic: of or pertaining to the habitat of free-floating or free-swimming organisms (plankton and nekton, respectively).

protozoea (e): that larval stage in the development of penaeids preceding the mysis in which propulsion is by thoracic appendages and there are no abdominal appendages (corresponds to the zoeae of other decapods in part).

sac-fry: the just-hatched larvae of fishes in which the external yolk sac is still present.

screening test: a preliminary test to determine the range of concentration within which lies the 96 hr TL_{50}.

standard length: the length of fishes measured from the mouth to the end of the peduncle.

static test: a test in which conditions were maintained constant throughout the test period.

sublethal: refers to a concentration of toxicant (or other adverse condition) less than the 96 hr TL_{50} which causes less than 50% mortality.

synergism: the interaction effect of two (or more) toxicants which is greater than the sum of the effects of each acting separately.

TL_{50}: median tolerance limit, i.e., the concentration of toxicant causing 50% mortality in a population at specified times, e.g. 96 hours.

vacuolated: possessing hollow spaces within cells.

yolk sac: that outpocketing of the gut containing yolk. Fishes generally have both an internal and an external yolk sac.

zoea (e): the planktotrophic larval stage of decapod crustaceans in which propulsion is provided by the thoracic appendages; shrimp-like.

* U. S. GOVERNMENT PRINTING OFFICE :1973—514-154/258